Pharmacogenetic markers of response to drugs used in the management of Inflammatory Bowel Disease

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Abstract

Background and Aims: The inflammatory bowel diseases (IBD) are chronic diseases that affect populations worldwide and impart considerable morbidity to those affected. Immunosuppression in IBD is effective but limited by side effects and non-response. We aimed to identify pharmacogenetic markers of response to commonly used immunosuppressive medication in these patients.

Methods: The influence of MTHFR 677C>T, MTHFR 1298A>T, ATIC 347C>G, TSER *2/*3 tandem repeat, TYMS 3'-UTR 6 bp insertion/deletion, SLC19A1 80G>A, AOX 3404A>G polymorphisms, 14bp ins/del polymorphism in the HLA-G gene and the IL-10 -1082A>G, -819C>T and -592C>A promoter SNPs were investigated on clinical response and side effect rates in 201 patients with Inflammatory Bowel Disease (IBD) treated with methotrexate (MTX). The HLA-G 14 bp ins/del polymorphism was correlated with azathioprine (AZA) response in 97 IBD patients. The IL-10 -1082A>G, -819C>T and -592C>A promoter SNPs on chromosome 1 were examined for an influence on the effect of the 14bp ins/del polymorphism in this group. The 14bp ins/del polymorphism was examined for an influence on susceptibility to IBD in 3148 individuals compared with 1330 normal controls. Stimulated PBMC from 14 normal individuals were incubated with MTX and 6-MP and expression levels of soluble HLA-G and IL-10 were correlated with genotype.

Results: A small positive correlation was seen for the *MTHFR677TT* genotype for clinical response and for the *ATIC347GG* for intolerance to MTX. The 14bp ins/del polymorphism strongly predicted clinical response to MTX (p=0.0016). The same influence was seen in 97 IBD patients treated with azathioprine (AZA) (p=0.001) and neither of these effects were affected by variant IL-10 promoter haplotypes. The described polymorphism did not influence susceptibility to IBD. Individuals with the 14bp del/del polymorphism expressed more soluble HLA-G after incubation with 6-MP (p=0.02) and MTX than carriers of the insertion allele. A dose effect was evident and carriers of the high producer IL-10 GCC promoter haplotype expressed significantly more IL-10 when incubated with 6-MP (p=0.04). This provides functional correlation of the genetic effect.

Conclusion: HLA-G genotype maybe a strong and predictor of response to MTX and AZA in IBD patients. These are preliminary findings and need replication in prospective studies.

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List of abbreviations used in this work

5′UTR	5′ Untranslated region
6-MP	6-mercaptopurine
7-OH MTX	7- hydroxy methotrexate
ADA	Adenosine deaminase
ALL	acute lymphocytic leukameia
AOX	Aldehyde oxidase
APC	Antigen presenting cell
ATIC	5-amino imidazole, 4-carboxamide ribonucleotide transformylase
AZA	Azathioprine
CARD	Caspase activation and recruitment domain
CD	Crohn's disease
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DHF	Dihydrofolate
DHR	Dihydrofolate reductase
FPGS	Folyl polyglutamate synthase
GALT	Gut associated lymphoid tissue
GWAS	Genome wide association study
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel disease
IL-10	Interleukin-10
IL-23R	Il-23 receptor
ILT2	Immunoglobulin like Transcript 2
INFX	Infliximab
INH	Isoniazid
Iso	Isoleucine
ITPA	Inosine triphosphate pyrophosphatase
KIR2 DL4	Killer-cell immunoglobulin like receptor 2DL4
LD	Linkage disequilibrium
Leu	Leucine
LPS	Lipopolysaccharide
LRR	Leucine rich repeat domain
MDP	Muramyl dipeptide
Met	Methionine
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
MTHFR	Methylene tetrahydrofolate reductase
MTX	Methotrexate

NF-KB	Nuclear factor kappa-light chain enhancer of activated B cells
NK	Natural killer cell
NOD	Nucleotide oligomerisation domain
RA	Rheumatoid arthritis
RFC	Reduced folate carrier
SNP	Single nucleotide polymorphism
TGF-β	Transforming growth factor-β
Th-1	T helper cell 1
Thr	Threonine
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPMT	Thiopurine methyl transferase
TR1	T regulatory cell 1
Treg	T regulatory cell
TS	Thymidylate synthase
TSER	Thymidylate synthase enhancer region
UC	Ulcerative colitis

Communications arising from this work

1. Sanderson JD, Smith M, Baburajan B, Ansari A, Lewis C, Marinaki T, et al. A Pharmacogenetic Index to Predict Non-Response to Thiopurines in Inflammatory Bowel Disease. Gut. 2009; 58:A95-A.
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1 Introduction

1.1 Pharmacogenetics

Pharmacogenetics or the study of the influence of individual genetic variation on drug effects has evolved considerably over the past 40 years. From a niche discipline in the 1950s, it has now found extensive application in many clinical trials. Whilst therapeutic practice has not yet been changed significantly by advances in the field, much is expected for the future.

It was a century ago that alkaptonuria and how it was affected by individual metabolic variation was described. Much of subsequent pharmacogenetics was often the reporting of extreme variant phenotypes. The serendipitous observation of the inter-ethnic variation in the side effect profile of primaquine during World War II is often regarded the first major pharmacogenetic influence on scientific investigation. A number of other observations followed in its wake during the 1950s.

The initially intriguing and seemingly inexplicable primaquine associated haemolysis seen in African American soldiers but not in their White American counterparts [1] was found to be due to genetic variation of the enzyme glucose -6-phosphate dehydrogenase (G-6-PD) [2]. Primaquine was an antimalarial drug given during World War II to American soldiers. Many African-American soldiers treated developed a haemolytic disease whilst white Americans were relatively unaffected. This deficiency is more prevalent in populations originating from areas with a high malarial infestation load and appears to provide a degree of protection against infection [3]. After the initial description many more polymorphisms have been described but like other pharmacogenetic markers, a functional assay of enzyme effect is preferred in the diagnosis [4].

Shortly after this, a pseudocholinesterase variant called butyryl- cholinesterase was discovered and credited with a prolonged duration of action of succinylcholine; a drug often used in general anaesthesia and was rarely (1:1000) complicated by a profound apnoea [5].

Similarly, on the theme of inter-ethnic variation in drug response, a description of a defect in the metabolism of isoniazid [6] followed. This was attributed to variation in the acetylation of INH by arylamine N-acetylytransferase-2 [7]. Two distinct metabolic phenotypes (rapid acetylators and slow acetylators) have been described worldwide with significant variation in frequencies among populations (50% among Caucasians, Asians and Africans; 20 % Amerinds) [8]. The

polymorphisms that lead to diminished enzyme activity are relatively recent discoveries [9]. Another important observation was the difference in metabolism of barbiturates (amobarbital) amongst Caucasians and Chinese individuals [10].

The antihypertensive debrisoquine [11] and anti arrhythmic sparteine [12] were found to have severe toxicity in certain patients and these effects were attributed to the already recognized lack of activity of the cytochrome P450 2D6. Subsequently, differences in the efficiency of metabolism of various medications dependent on genotype were noted and could be classified into poor metabolism, intermediate metabolism, rapid metabolism and ultra rapid metabolism [4, 13]. A variety of drugs are metabolized by these pathways and the effects of the various phenotypes on drug response or toxicity differ according to whether the drugs are activated or inactivated (4).

The traditional approach to pharmacogenetics has been based on clinical observations in affected families or in the general population that then leads to an identification of variation in single genes coding for drug metabolism enzymes. Advances in genetics have revealed the potential of the newer field named Pharmacogenomics. With high-throughput technology, large numbers of genetic polymorphisms can now be detected accurately and rapidly. In a wide ranging model, that includes many genes and their interactions, Pharmacogenomics is now a field that encompasses genetics, pharmacodynamics and pharmacokinetics. These are now frequently a part of large clinical trials and important genetic associations with individual drug response may be forthcoming [14].

Despite all of these discoveries and of many genetic influences on drug metabolism, true clinical application has been rare and this is unlikely to change in the immediate future. However, one of the best examples of successful translation of pharmacogenetics to clinical practice is the impact of genetic variation in the activity of thiopurine-s-methyl transferase (TPMT). This is a central enzyme in the metabolism of azathioprine/6-mercaptopurine, two drugs used commonly in cancer chemotherapy and inflammatory diseases. However, even here it is the phenotypic assay of activity that has found widespread use rather than genetic testing. A more detailed review of this follows. The search for other markers continues.

1.2 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract. The two main clinical subtypes are Crohn's Disease (CD) and ulcerative colitis (UC). Although similar in many respects, significant differences exist in the cytokine milieu at the mucosal level between these diseases which are conventionally divided along Th1, Th2 and the emerging Th17 lines. Modern genetic association studies have identified various single nucleotide genetic polymorphisms probably responsible for important changes in the interactions between bacteria and host innate immune mechanisms [15]. Despite impressive strides in unravelling the genetic basis for CD and to a lesser extent UC, the direct clinical applications of these advances have been slow. The investigation of pharmacogenetic markers allowing for optimal therapy of these diseases remains an important but largely unfulfilled ideal.

It is likely that the diseases are the consequence of a complex interaction between the intestinal bacterial flora and the innate immunity, influenced by the host genotype. In effect; a dysfunctional immune response, by a genetically predisposed individual to what would, otherwise have been a normal bacterial challenge.

Samuel Wilks described UC in 1859 [16] and it was not until 1932 when Burrell Bernard Crohn gave us a remarkably accurate description of what he and his colleagues termed "regional enteritis", that Crohn's disease was recognised. Much of the anatomical and pathological features noted at the time are familiar to us in current practice. It is encouraging that we have moved on from the palliative and supportive management that was the fate of patients not suited to surgical resection [17]. It would appear that CD is relatively new. The diseases are common and 1.4 million people in the US and 2.2 million people in Europe are affected. Since its first description, the incidence has been rising in the western world although some suggest that these rates are starting to stabilise [18]. However, there has been no such effect in Southern Europe, Africa or Asia where rates continue to rise.

1.2.1 The Etiopathogenesis of IBD

1.2.1.1 Ethnicity and geographic differences

There are clear differences in the racial predilection to the inflammatory bowel diseases. The augmented risk among the Ashkenazi Jewish population in Europe, USA and Cape Town is well known [19]. However, clearly there are also other influences. Migrant populations gradually take on the disease predilection of the pre-existing residents but it remains less prevalent. This effect has been seen in all high prevalence areas and include the Hispanic and Asian populations in the United States [20], The Bangladeshi population in East London, UK [21], the Chinese population in mainland China [22] and the more recently immigrant of the Jewish population in Israel [23, 24]. Being a first-generation Canadian is protective against the development of CD [25] and immigrants from the UK in New Zealand have a greater risk of both UC and CD than the longer term residents [26].

1.2.1.2 Smoking

In 1982, Harries et al. first noted that smoking was rarely reported among sufferers of UC [27]. Some time later the opposite effect was noted among CD patients and this remains the current view on the effect of tobacco on the natural history of IBD. However, the pathophysiological basis for these differences remains obscure. The odds ratio for developing UC is lower in a current smoker compared with lifetime non-smokers (OR= 2.41, CI 0.34 to 0.48) [28]. Smoking cessation is associated with more relapses, hospital admissions, oral steroids and immunosuppressants among sufferers of UC. The risk of surgery was not different [29]. The opposite is true in CD, where the odds ratio is 2 (CI 1.65- 2.47) when current smokers are compared to those who have never smoked [28]. In a similar vein, the risk of clinical and surgical recurrence was lower in non-smokers than in smokers and the risk is dose-dependent [30]. Smoking cessation diminishes the need for immunosuppression and other medical intervention to levels similar to those enjoyed by lifelong non-smokers [31]. The fascinating report that smoking appears to influence disease concordance in siblings of affected individuals, deserves mention. CD occurred in the smoking and UC in the non-smoking relatives of the affected IBD patient (OR=10) [32].

1.2.1.3 Other aspects of the environment

There has been a rapid increase in the inflammatory bowel diseases around the world; and previously low risk populations are displaying increasing disease incidence and prevalence.

1.2.1.4 Hygiene hypothesis

Increasing environmental hygiene is associated with increasing predispositions to autoimmune and inflammatory conditions. It is recognized that most autoimmune conditions are associated with imbalances between Th1 and Th2 related cytokines. These have reciprocal effects on each other and are influenced by infections. The ensuing production of regulatory T-cells (T_{reg}) leads to "bystander suppression". It is likely that this is mediated by Interleukin-10 (IL-10) and Transforming growth factor-β (TGF-β) from CD25+ T-cells [33]. In a similar fashion, allergies and autoimmune conditions appear to be associated [34].

Environmental factors that may favour a high infectious burden such as crowded living conditions, and consumption of contaminated foods and a lack of potable water may confer low risk of IBD. This is particularly true for CD. It is proposed that limited exposure to environmental antigens impairs immune maturation and results in a dysfunctional immune response on exposure at a future time [35, 36].

1.2.1.5 Bacteria

The human intestine is sterile at birth but then very rapidly becomes colonized by bacteria from the mother's intestinal and vaginal flora [37]. The ecosystem of the intestine is very dynamic but some microbial members are long-term "residents". Despite the large environmental species diversity among microbes, the intestinal environment clearly mandates a more restricted population. Selection pressures reduce the bacterial diversity of the planet (over 55 divisions) to just the Bacteroidetes, Firmicutes and one member of Archaea [38].

However, there is considerable strain variation; and is reminiscent of patterns seen where a few successful early colonists give rise to a variety of descendants. This is evident in instances of

extreme selection pressure and as been noted in Helicobacter pylori populations around the world [39], and in current theories about the origin of human populations [40].

Commensal bacteria clearly influence absorption, mucosal integrity and angiogenesis [41]. The microbial population in the gut can provoke IBD but probably not due to direct pathogenicity [42]. Immune deficient mice do not have a different intestinal bacterial population to immunocompetent animals [43]. An inadequate or inappropriate immune response to commensal bacteria can drive the inflammatory cascade in IBD. In immune competent mice, antibiotics can control colitis and transfer of activated CD4+ T-cells into immune deficient mice causes colitis. This is not seen in germfree subjects [44]. IBD patients have an enhanced immune responsiveness to bacterial antigens, with a concurrent loss of tolerance. It is likely that this is influenced by commensal bacteria [45]. This concept of tolerance is expanded in a subsequent section.

1.2.1.6 Genetics

Family members of a patient affected by IBD have a higher risk than that of the background population of being affected as well. Indeed, a positive family history is the greatest risk factor for developing IBD (both UC and CD) [46]. The genetics of IBD are complex and cannot be approached with simple Mendelian concerts of monogenic inheritance. They are polygenic diseases, affected by several gene mutations that interact with each other and influence a number of phenotypes [47].

Valuable information has been gleaned from twin studies that by their very nature, overcome important difficulties in adjusting for the contribution of environmental factors to the estimation of congenital risk. These studies that cumulatively report on 322 patients have estimated that monozygotic twins have a concordance rate of 37% for CD and 10% for UC [48-50]. These observations also demonstrate the important role that environment has in modifying the genetic risk, as otherwise, the concordance among twins may have approached 100%.

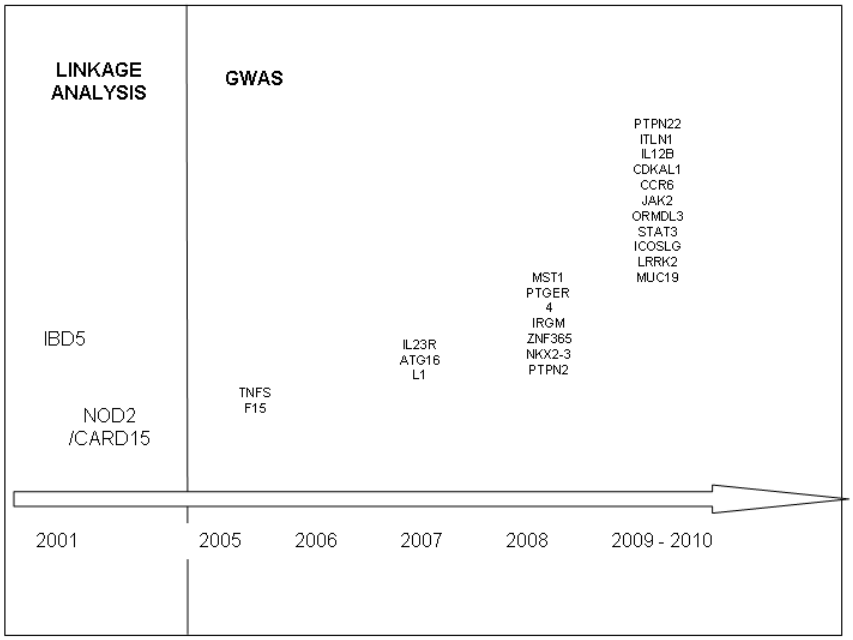
There have been numerous attempts to quantify this risk in more detail. Unfortunately these classical large-scale referral hospital and population-based studies are limited by inconsistencies in phenotypic description, the lack of adequate age adjustment, the contribution of high risk populations (such as Jewish people), variation in the time point of assessment of what is a dynamic disease, and the chances of dual reporting of disease location [51].

However, the risk of both CD and UC is higher than that of the background population. There does not appear to be greater concordance for disease location or disease nature among twins compared with other, non-twin siblings [52]. Across all studies, the estimate of λs (the risk to siblings of a patient developing the disease compared with a member from the general population) is 20–35 for CD and 8–15 for UC [53].

Major advances in techniques that have found application in large-scale population genetic studies have paid off handsomely in identifying genetic differences between populations with CD, and those who don't. Whilst the polygenic basis of the disease has limited the application of these findings in clinical diagnosis or monitoring of the disease state; they have led to an explosion of knowledge in the immune pathogenesis of disease [51].

Early investigators used candidate gene studies, looking at polymorphisms within genes of known function that were likely to be important in IBD. Since 1996, a number of 'hypothesis-free' genome scans have been performed in IBD to identify regions of the genome likely to contain disease susceptibility genes. A systematic screen of genetic markers distributed across the human genome is made in a large number of affected individuals. This technique is more powerful when related individuals similarly affected by a disease are included. When affected siblings demonstrate more shared alleles than expected by chance, linkage is identified. Where such regions are replicable in independent family panels they merit further investigation, fine mapping by association study or positional candidate gene analysis.

In a number of full genome scans published for IBD, 9 strong candidate regions designated IBD 1-9 have been identified. A recent meta-analysis of 10 GWAS studies has demonstrated linkage on chromosomes 1, 2, 3, 5, 6, 7, 16, 17 and 19 [54]. An excellent example of the candidate gene approach in IBD has been the studies of the HLA complex on the short arm of chromosome 6. More recently much attention has focused on genome scanning studies, but the process is beginning to come full circle with the acceptance that a powerful strategy for disease gene identification is to combine both approaches. Such genes are implicated both because they map to a region of linkage identified in a genome scan, and also by their expression pattern or function. (Figure 1-1)



(Adapted from Budarf et al 2009)

Figure 1-1 The influence of GWAS on the success of susceptibility genetics studies in IBD

1.2.1.6.1 IBD1 (Chromosome 16) NOD2/CARD15

The IBD1 locus on chromosome 16 has been extensively replicated and mutations in the NOD2 gene influence some aspects of the pathogenesis of CD but not UC. NOD2 was reported simultaneously by Hugot et al [55] and Hampe et al [56] who applied classic techniques of LD mapping and identified one frameshift and two missense mutations, and Ogura et al [57] who used a candidate gene approach. Average relative risks for CD in genotypes containing zero, one or two of the variants were 1, 3 and 38 respectively in the French study. This finding has now been replicated in many panels around the world, with recent data suggesting that NOD2 mutations are only associated with ileal inflammation, and not CD where the colon alone is inflamed [58]. Double dose mutations (homozygous or compound heterozygous) particularly increase the risk of early onset, penetrating disease of the ileum. Although over 30

polymorphisms are present within this gene, three single nucleotide polymorphisms (SNP) form 80% of the mutated alleles [59]. There is variation among populations in the contribution these polymorphisms make to susceptibility to, and the phenotype of CD. In high prevalence areas such as the UK and USA, up to 40% of patients have these mutations [59]. This effect is absent amongst East Asian populations [60] and is less evident in Scotland and Ireland [61].

The protein encoded by this gene is present in Paneth cells [62], monocytes and tissue macrophages [63] and intestinal epithelial cells. Its primary role is in the recognition of bacterial pathogens [64], and a rare autosomal dominant disorder called Blau syndrome affecting NOD2 is manifest as familial granulomatous arthritis, iritis and skin granulomas in the affected individuals. The parallels with IBD are obvious.

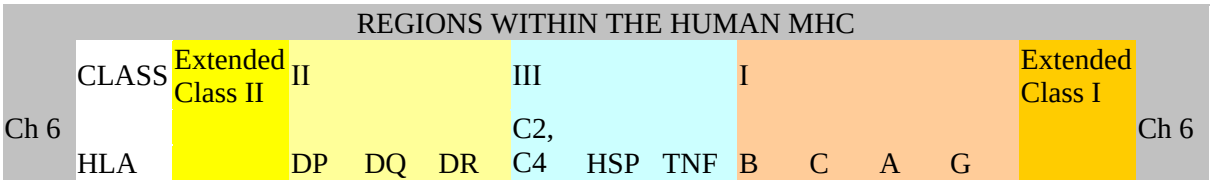
The mutations identified affect the leucine rich repeat domain (LRR), which plays a role in sensing bacterial muramyldipeptide (MDP) lipopolysaccharide and regulating NF-κB. Unlike *in vivo* findings in inflammatory bowel disease, the mutant *NOD2* gene appears to correlate to low levels of NF-κB [64] in knockout mouse models. A recent knock in mouse model study of the *3020insC* polymorphisms has revealed findings consistent with clinical scenarios in human IBD [65].

1.2.1.6.2 IBD3 (Chromosome 6)

Attention to the significance of the HLA region in IBD was first drawn by Japanese studies in which HLA-DR2 was associated with UC [66]. However this allele does not appear to be associated with IBD in Caucasian populations.

In a Caucasian panel, Satsangi et al [67] identified association between *HLA-DR3*, *DQ2* and extensive UC particularly in females, and *DRB1*0103* in both severe disease requiring surgery and extra-intestinal manifestations. This is a rare mutation occurring in 0.2% to 3.2% of the population. In CD, the *HLA-DRB1*0701*, a common allele has been associated with ileal disease particularly in individuals who were unaffected by CARD mutations [58, 68]. The HLA region

clearly influences extra intestinal disease in IBD. There is a large joint inflammatory oligo-arthropathy associated with the *DRB1*0103* allele, and a small joint arthropathy, associated with *HLA-B44* [69].



(Adapted from Ranganathan, 2005)

Figure 1-2 A diagrammatic representation of the gene map of the extended Major Histocompatibility Complex

Although original association data for CD overall was inconsistent at the HLA locus, attention has recently been re-focused by the results of linkage studies. These strongly suggest a role for this region in CD as well as UC, and there is consistency now both for particular HLA alleles and TNF promoter polymorphisms [58]. The latter have shown replicable association in Japanese and Caucasian populations. In a recent study van Heel et al demonstrated association between IBD and a functionally significant TNF promoter polymorphism which appears to enhance binding of NF-κB [70].

Despite the replication, it is still unclear exactly which gene or genes on chromosome 6p contribute to IBD. This is because numerous immuno-active genes map here and there is extensive linkage disequilibrium (LD) in this region. Strong LD means that this is an area where few cross-overs tend to occur at meiosis, and thus association will be seen with 'hitchhiking' polymorphisms widely distributed to either side of a disease causing mutation.

1.2.1.6.3 IL23R

Original evidence for linkage between 1p and CD came from Chicago and John's Hopkins, USA [71]. Individual variation in the *IL23R* gene is associated with a variation in susceptibility to both CD and UC [72]. These findings have been extensively replicated [73, 74]. A rare variant in this gene appears to offer protection against IBD and functional data has since then demonstrated the importance of the IL-23/Th-17 pathway in IBD pathogenesis. Other variants are also present but the effects are less obvious. The IL-23/Th-17 pathway has been described in greater detail elsewhere in this document.

1.2.1.6.4 The genetics of susceptibility to UC

Most initial studies were focused on CD due to the greater degree of heritability that has been attributed to it. As GWAS have become more widely available, the techniques have found application in UC. The results are intriguing and the two diseases are becoming increasingly separated at the genomic level [75]. A number of loci have been identified, of which the most interesting would be the ECM1 locus [72] and the *IL-10* [76] and *IL-10 R* genes [77].

It is also of particular interest that the genes influencing the innate immune pathway are not implicated in UC, in particular, *NOD2, ATG6L1,* and *IRGM*. It is possible that the pathogenic mechanism in UC does not involve as large a bacterial contribution as in CD [78].

Substantial progress has been made in the past 10 years in understanding the genetic basis of IBD and genetic studies may yet, provide a quantum leap forward in understanding the pathogenesis of IBD, which will drive the development of rational new therapies.

1.2.1.7 Immunology

1.2.1.7.1 The normal Intestine and Innate Immunity

The antigenic diversity of the human intestine is vast, and by far the largest contribution to this comes from the resident and occasionally transient bacterial flora. There are over 100 trillion microbes and the vast majority reside in the colon. The density of the microbial population here is the highest recorded for any microbial habitat [79]. There has been increasing recognition that the intestinal microbiota plays a critical role in the maintenance of immunological homoeostasis in the gut. CD4+ T-cells maintain the peripheral immune surveillance of antigens. Following selection in the thymus, effector and regulatory CD4+ T-cells (T_{regs}) specific to bacterial antigens are focussed in gut associated lymphoid tissues (GALT) in the small and large intestines [80].

The system is predominantly one of immune tolerance and regulation. One only needs to consider the effect of bacterial capsular lipopolysaccharide A (LPS A) that is a component of over 5% of the bacterial population of the human intestine. It can stimulate a large fraction of the T- cell population [81]. Indeed, if these bacteria were presented in a parenteral manner, a catastrophic inflammatory response would ensue. Instead, in the healthy intestine, CD4+T helper cell and T_{reg} mediated tolerance predominates [82].

The epithelium separates the intestinal lumen from the immunologically active lamina propria. The secretion of antimicrobial defensins by Paneth cells [83]; and mucus perpetuate this barrier. The intestinal epithelial cells (IECs) produce anti-inflammatory cytokines like IL- 25, IL-10 and TGF β. Their downstream effects lead to sequestration of mucosal antigen presenting cells (APC), and the down-regulation of MHC II, adhesion molecules and pro-inflammatory cytokine such as IL-6, IL-23, or IL-12 [80, 84].

Despite this, the peripheral T_{reg} population is influenced by the intestinal bacterial environment [85]. Clearly the intestinal epithelium is not impermeable not least because it provides a route for nutrient absorption. There are two routes; one, a transcellular route with specific membrane pumps and channels and a separate para cellular route that is policed by tight junctions, occludin and claudin proteins [86].

Epithelial pattern recognition receptors that identify microbial components like LPS, RNA, and methylated DNA. Fifteen toll like receptors (TLRs) taken together are capable of recognizing most microbial molecular patterns including commensal bacteria [87]. When they do, they trigger an intracellular immunological response [88]. They can increase or decrease the function of T_{reg} cells [89] and lead to enhanced survival and accelerated induction of T effector cell responses. TLRs are also expressed on CD4+T cells (effector and regulatory) and stimulate adaptative immunity.

Cytoplasmic NOD 1 and 2 proteins are expressed in APC and in small intestinal Paneth cells [62] and may provide an additional defence mechanism. It is activated by bacterial peptidoglycan through MDP [90]. Expression is augmented by inflammatory cytokines [91] and NOD 2 mutation increases NF-κB and IL-1 β activity [65].

Dendritic cells (DC) sample bacteria by dispatching probes into the intestinal lumen through tight junctions between epithelial cells. When DCs detect pathogenic bacteria, they mature, and induce immunity. DCs are important in balancing immune responses against pathogenic bacteria and tolerance towards commensal bacteria. They achieve this by expressing the entire spectrum of TLRs and NODs [84, 92].

1.2.1.7.2 Adaptative Immunity

The gut mucosa is home to a large lymphocytic population. These include T-cells, B cells and natural killer (NK) cells. Villous microfold cells transmit antigenic material to Peyer's patches in the small intestine as one of the key elements in the adaptative immune system. This is comprised of two populations of lymphocytes: the B lymphocytes and the T lymphocytes.

All lymphocytes originate in the bone marrow. Subsequent T-lymphocyte maturation occurs in the thymus and B lymphocyte maturation in the bone marrow. In the periphery, lymphocytes are localised in mucosa associated lymphoid tissue (MALT) and to smaller extent in the lymphoreticular system. B lymphocytes possess immunoglobulin receptors that recognize intact antigens and by the secretion of immunoglobulins, activate the humoral arm of immunity. They act against extracellular pathogens in correlation with the complement system and phagocytes.

The T-lymphocyte is responsible for the cell mediated arm of immunity that responds to intracellular pathogen such as viruses. The T-lymphocytes also regulate B-cell responses. The T-lymphocytes possess T-cell receptors (TCR) that recognize antigen bound to the major histocompatibility complex (MHC) and APCs. The effective activation of T cells requires specific mature APC s (dendritic cells or macrophages with high levels of MHC class II molecules and co-stimulatory molecules). If any other APCs are the presenting cells, anergy or tolerance may result [93].

These populations of T-cells are recognized.

- Effector CD4+T cells
    - Type 1 helper T-cell (Th-1 cells)
    - Type 2 helper T-cell (Th-2 cells)
    - Th-17 cells
    - T regulatory cells(T_{regs}) (CD4+ CD25+ FOXP3+)

- Cytotoxic CD8+T cells
    - Cytotoxic T-lymphocyte (CTLs)

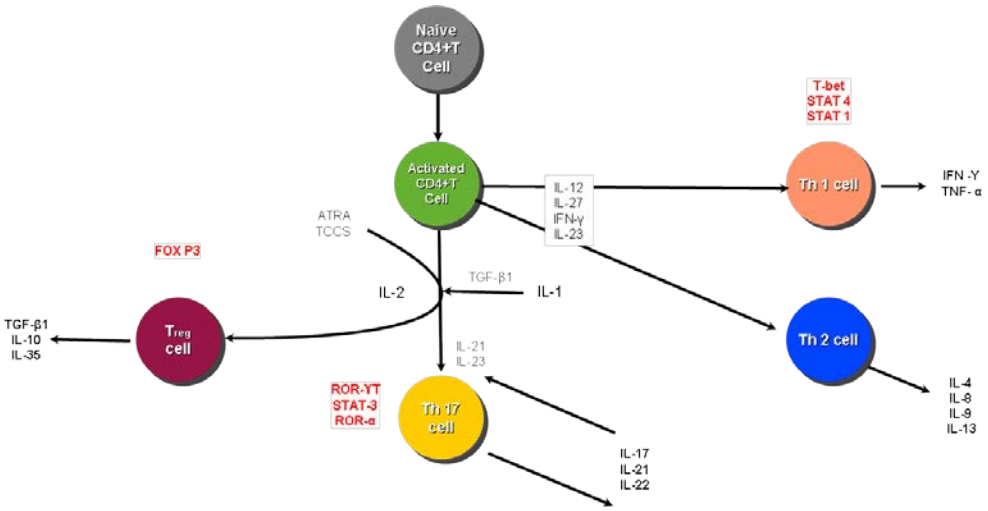
$CD4^+$ T cells

These T-cell subsets have distinctive cytokine expression profiles and responses to specific transcription factors. The uncommitted $CD4^+$ T cell (Th-0) cell is influenced by a locally existing pattern of cytokines that steers the T cell population towards a Th -1 or Th- 2 phenotype. These secrete individual and exclusive cytokine profiles and mediate distinct effects. The Th-1 cellular population promotes cell mediated immunity by macrophages and antibody dependent cell mediated cytolysis. The Th-2 population provides mucosal immunity by stimulation of mast cells, eosinophil differentiation, IgA synthesis (antibody and allergic responses). The two cellular populations influence each other by important feedback mechanisms. Monocytes secrete IL-12 which stimulates the secretion of IFNγ by Th-1 cells. This upregulates IL-12 receptors (IL-12R) inhibits Th-2 and further augments the inflammatory response. An early secretion of IL-4 favours a Th-2 profile characterised by the secretion of IL-10. This inhibits IL-12 secretion by monocytes

and augments IL-4 production by Th-2 cells. The IL-4 inhibits the expression of IL-12R and shifts the balance towards a Th-2 phenotype (Figure 1-3).

Other T cells

These phenotypically distinct cells can be cytotoxic (CTL: cytotoxic T lymphocyte) or suppressive $CD8^+$ T_{regs}. The cytokine profiles secreted can be very similar to those of the $CD4^+$ T cells. A number of other T cells are recognised and include Natural Killer cells (NK). These are capable of initiating T cell responses by secreting IFN-γ and IL-4 and regulating dendritic cell responses by secreting IL-10. They are obviously an interface between the innate and adaptive pathways.



Identifying markers: Red font, secreted cytokines: Bold black font, influencing cytokines: normal black font

Modified from Male et al., Immunology, 7th edition, Mosby 2007

Figure 1-3 An overview of T cell differentiation

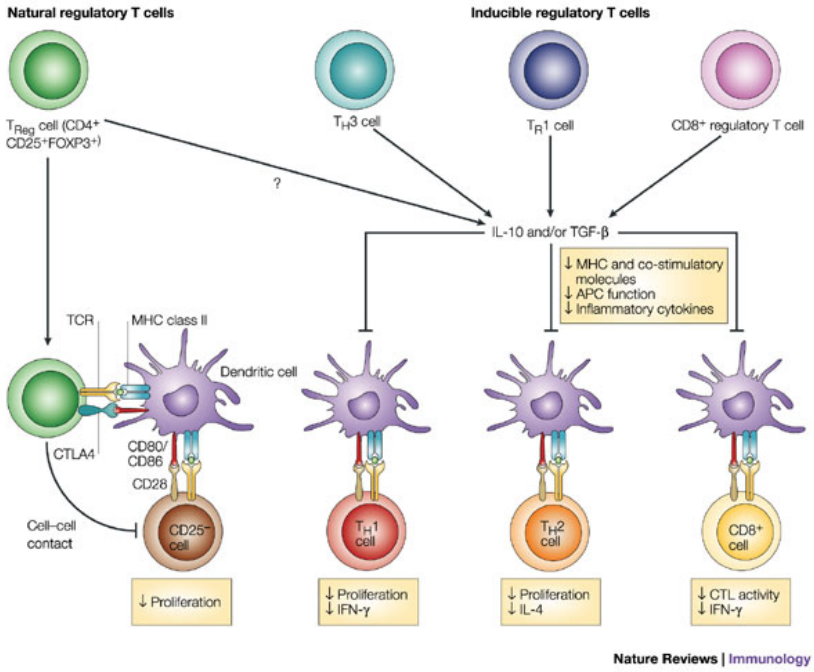
T regulatory Cells (T_{regs}) and Tolerance

The activated inflammatory cascade has the potential for unchecked destruction and mechanisms are in place to limit this. A subset of T cells is $CD4^{+}CD25^{+}$ and is regulatory or suppressive in function. Precise characterisation of these cells has been difficult as they do not express distinctive cell surface markers. However, their discovery has developed the concept of tolerance.

Two mechanisms of tolerance have been proposed and “recessive” tolerance is a means of inducing apoptosis of self reactive thymocytes that escape clonal deletion. These cells become anergic. In the periphery, a similar mechanism exists and appears to be mediated by natural regulatory T cells. Simultaneous engagement (by cell to cell contact) of the T cell receptor and CTLA4 by CD80 and CD86 ligands on antigen presenting cells (APC) such as dendritic cells (DC) [94] reduces proliferation of naïve and activated T-cells.

This process of “recessive” tolerance is augmented by specialised cells that suppress self reactivity and this is “dominant " tolerance. These induced T regulatory cells may be antigen specific or non-specific and express the $CD4^{+}$ $CD25^{+}$ phenotype typical of all active T lymphocytes. Importantly, they differ from others in their unique cytokine-expression profile.

Non-antigen specific T cells are $CD8^{+}$ T Cells, γδ T Cells and $CD4^{+}$ T cells. Of these, $CD4^{+}$ T_{regs} secrete IL-10 or TGF-β and this differential expression allows their classification into two cell populations. T_R 1 cells produce high levels of IL-10 [95], T_H 3 cells produce large quantities of TGF-β [96] and, without cellular contact dependent mechanisms; these cytokines mediate their suppressor effects. The T_R 1 cells suppress Th -1 cell responses and Th -1 cell mediated auto immune diseases. IL-10 inhibits the production of TNF and IL-12 by DCs and macrophages and the addition of IL-10 antibodies can reverse the suppression [97]. TGF-β secreted by T_H 3 cells also inhibits T_h 1 cell responses by inhibiting T-bet transcription and IL-12 receptor expression [98]. Furthermore, TGF-beta1 induces Smad4, which then binds to and activates the IL-10 promoter and subsequently increases IL-10 expression [99]. Clearly the T_R 1/ T_H 3 cell populations are involved in an interdependent immunosuppressive effect (Figure 1-4).



CD4+CD25+FOXP3+ (forkhead box P3) natural regulatory T cells (TReg cells) inhibit the proliferation of CD25- T cells by cell–cell contact. These then express cytotoxic T-lymphocyte antigen 4 (CTLA4), which interacts with CD80 and/or CD86 on the surface of antigen-presenting cells (APCs) such as dendritic cells (DCs), and this interaction delivers a negative signal for T-cell activation.
T regulatory 1 (TR1) cells, T helper 3 (TH3) cells and CD8+ regulatory T cells, secrete IL-10 and/or TGF- which inhibit the proliferation and subsequent cytokine production by TH1 cells, TH2 cells and CD8+ cytotoxic T lymphocytes (CTLs)

Figure 1-4 T_R1 and Th-3 differentiation and the influence on tolerance

Reproduced from Mills et al. [97]

Antigen specific T_{regs} include $CD8^{+}CD28^{-}$ T cells that express Foxp3. They suppress the antigen – specific response of $CD4^{+}$T cells by cell to cell contact [100], reducing IL-2 secretion, down regulating co-stimulatory molecules such as CD80 and CD86 and up reregulating inhibitory receptors such as ILT3 and ILT4 on APCs [101].

As briefly described before, the maturity of DC in their antigen presenting role to T cells influences the response. Immature DC s induces an anergic response. However mature DCs are also capable of inducing tolerance by increasing regulatory $CD4^{+}CD25^{+}$ T cells [102]

1.2.1.7.3 The immuno-pathogenesis of inflammatory bowel disease

It is likely that a predisposition to abnormal intestinal permeability exists in individuals with a family history of CD and in those with a *CARD*15 3020 ins C mutation [103]. There has been one report of an individual with abnormal intestinal permeability going on to develop CD [104] and these abnormalities have been noted in both in inflamed and non-inflamed areas of the bowel, in CD and UC [105]. A co existing defect in the innate immunity is most likely to be the next step. It may involve the TLRs. TLR4 is significantly up-regulated in CD and UC and TLR3 is down regulated in CD but not in UC [106]. In the injured mucosa, TLR5, usually expressed basolaterally, is exposed to bacterial flagellin and subsequently activated [107]. DC's respond inappropriately to commensal bacteria with a Th-1 response normally reserved for pathogenic bacteria. [108]. The DC's have a mature, activated and pro-inflammatory phenotype [109] rather than the immature, tolerogenic phenotype [110].

There is a persistent failure of apoptosis in activated, reactive, mucosal T-cell populations [111] and the repeated activation of memory T-cells that perpetuate inflammation. Furthermore, the intestinal epithelial cells themselves activate T-cells through a variety of pathways including increased MHC expression in the presence of inflammatory cytokines [112] and some others that arise in injured colonic mucosa [113].

However, major developments in the understanding of CD4+ T-cell and T_{reg} cell function in IBD in recent years have further enhanced our understanding of pathogenesis. CD4+ lymphocytes are key to the development of IBD. They are increased and activated in the intestinal lamina propria

[114] and are dependent on the presence of intestinal microflora to generate inflammation [115]. Until recently, CD was thought to manifest a predominantly Th-1 phenotype, mediated by the sequential activation of signal transduction cascadesin activated T-cells (STAT-1 and STAT-4 by IFN α, IFN β and IFN-γ followed by the induction of the T box transcription factor (T-bet) and IFN-γ [116]. Th-1 mediated immune responses are evoked on exposure to an intracellular pathogen, such as a virus or bacterium. The immune response localises the infectious agent and by the secretion of IFN-γ and TNF-α or the differentiation of cytotoxic T-lymphocyte leads to microbial killing.

The evidence for this is powerful, and includes

1. The increased numbers of CD4+ T-cells that express IFN-γ and T-bet in the inflamed colon [117, 118].
2. Increased serum IFN-γ in CD patients but not in normal controls [119].
3. Increased expression of IL-12 receptor and IL-18 receptor on lamina propria CD4+ T-cells of CD patients and subsequent increased production of IFN-γ when activated with the corresponding cytokine [120].
4. Increased levels of IL-12 in the inflamed mucosa [121] and the efficacy of IL- 12 antibody therapy in CD [122].

Since high levels of IL-17 were reported in IBD in 2003 [123], some of these concepts have been revised. They have been significantly influenced by recent studies on Th-17 CD4+ lymphocytes which are found in large numbers in intestinal mucosa of patients with CD and UC [124]. They are found in the lamina propria of patients with UC and throughout submucosa muscularis propria of patients with CD; namely areas that display characteristic pathological changes. Th-17 cytokines up regulate inflammatory cytokines and matrix metalloproteinases (MMP). These amplify T-cell effector pathways and cause significant tissue damage [125, 126]. When Th-17 cells reactive to intestinal bacteria are transferred into immuno-deficient mouse recipients, the colitis induced is of a comparably greater severity than transfers of Th-1 cells.

Th-17 cells are typified by the expression of the transcription factor RORγt [127] which is, in turn, dependent upon STAT-3 expression. TGF-β and IL-6 commit naïve T cells to Th-17 cell differentiation. The stimulated cells secrete IL-17 and IL-22 [128] and IL-10 and mediate a homoeostatic intestine. As the Th-17 cells mature, they acquire responsiveness to IL-23 [129].

IL-23 receptor [130] stimulation with IL-23 promotes expression of IL-17 and pro-inflammatory cytokines, but not the anti inflammatory IL-10 [131]. IL23p19 monoclonal antibody inhibits colitis and causes depletion of the co- transferred Th-17 effector cells. [132]. The inflammatory effects of IL-17 are apparently critically influenced by IL-23 [133].

In UC, in which IFN-γ levels are not increased, the situation is different. Based on this, a Th-2 hypothesis was proposed. However, there are significant departures from previously recognised patterns. Th-2 cells are rarely seen in the normal intestine and have a role in coordinating the response to helminthic infections. In UC, IL-4 levels are lower than expected but IL-5 levels are heightened. Animal models are somewhat limited in their similarity to human UC but it is useful to note is that an IL-4 dependent early phase of intestinal inflammation is superseded by an IL-18 dominated late phase.

1.2.1.8 HLA-G

Previous data have suggested strong roles for the highly polymorphic human leucocyte antigen (HLA) region, located on the short arm of chromosome 6 in susceptibility to IBD and in disease phenotype. It is clear that the HLA region contains genes that determine susceptibility to IBD [134, 135], that there are differences in associations between these two diseases, that ethnic groups differ and that some of these markers may have clinical significance in predicting disease course and the development of complications [136].

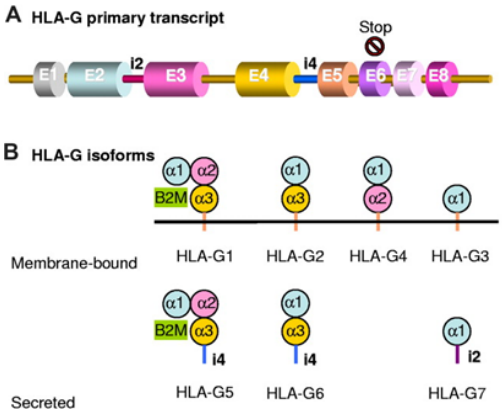
The primary role of the HLA molecules is to transport peptides to the T-cell antigen receptor and present these peptides for recognition and induction of T cell responses. Three classes are recognised and differ in their roles. Class I antigens are the products of the HLA-A, B, C, E, F and G genes. They also include pseudogenes (HLA-H, J, K, L, P and V). Class II antigens are the products of are HLA- DR, DQ, DP, DM and DO genes [137]. The Class III region is located between the other two and contains over 20 genes coding for a variety of peptides (Figure 1-2). The gene enjoys a wide range of genetic polymorphism and extended haplotypes are nearly unique for an individual. There is, on the other hand, very significant LD and the co existence of certain haplotypes can be predicted with great accuracy.

1.2.1.8.1 Biology

HLA-G is a non-classical class I antigen and that contrasts from classical HLA. The most remarkable of these is the extremely low level of genetic polymorphism (8 protein variants versus over 500 for HLA-A and HLA-B). The tissue distribution is highly restricted with only a few privileged sites demonstrating expression such as the human trophoblast, thymic medulla, cornea and pancreatic islets [138]. HLA-G expression is inducible in cancers, transplanted grafts, inflammatory diseases and viral infections [138]. The effects of the HLA-G molecule vary in these areas and whilst in the semi-allogeneic foetus, a survival benefit is evident, in malignant tissues, the protein expression leads to aggressive disease [165]. Unlike classical HLA, the molecule enjoys a profound deficiency in eliciting immune responses [140] and like classical HLA, a particular efficiency in suppressing them. HLA-G is up-regulated in monocytes and T-lymphocytes in HIV patients and in patients with CMV infections [141, 142] and may be a key defensive strategy for escape from immune surveillance.

Like classical HLA, there is a primary transcript. Here, it has as stop at exon 6 after the coding sequence for the trans-membrane tail. On alternate splicing, 7 isoforms are generated. These are membrane-bound (HLA-G1, G2, G3, G4) and soluble (HLA- G5, G6, G7) [143]. HLA-G5 is considered the soluble form of HLA-G1 and the extracellular segments of the two are identical and very similar to classical HLA. There is a heavy chain with three globular proteins linked to β2-microglobulin. Truncated forms are created by the excision of exons coding for some of these proteins and soluble forms are created by the translation of intron 4 or 2 [138] (Figure 1-5).

Of these forms, HLA-G1 may be the only one expressed at the cell surface [144]. However the molecule is not essential for maintenance of a viable pregnancy as homozygotes for this allele of the null HLA-G allele *HLA-G *0105N*, demonstrate [145]. They do not express HLA-G1, G5 or G4. Instead, HLA-G2 and G6 are seen [146] and appear to protect against NK cytolysis [147]. The soluble forms (sHLA) are detectable in body fluids [148] and decreased sHLA-G is observed in pre-eclampsia and preterm placental abruption [149]. Increased sHLA-G levels are associated with an improved pregnancy outcome after *in vitro* fertilisation (IVF) and improved graft acceptance after cardiac and liver-kidney transplantation [150, 151]. Membrane-bound and soluble forms of HLA-G inhibit Natural killer (NK) cells, cytotoxic T-lymphocytes [152-154], DC maturation and induce CD4+ T regulatory cells (T_{reg}) differentiation. These mechanisms may prolong graft survival [155].



Reproduced from Carosella et al [138]

Figure 1-5 The HLA-G primary transcript and the effect of alternate splicing and translation on the expression of HLA-G isoforms.

Table 1-1 The effects of HLA-G on effector cells and the receptors that mediate these functions

Expressing Cells	HLA-G functions	ILT 2	ILT4	KIR2DL4	CD8
NK CELLS	Inhibit Cytotoxicity	+		+	
	Suppress proliferation	+			
	Apoptosis				+
	Inhibit trans endothelial migration	+			
CD8+ T cells	Inhibit Cytotoxicity				
	Suppress proliferation	+			
	CD8+T_{reg} differentiation				
	Apoptosis				+
CD4+ T-cells	Inhibit alloreaction	+	+		
	Suppress proliferation	+			
Monocytes		+	+	-	-
DC	Inhibit maturation and induce T_{regs}	+	+	-	-

Adapted from Carosella et al

HLA-G acts on a number of inhibitory receptors that have site and cell specificity. ILT2 is widely expressed but ILT4 is myeloid specific, expressed only by monocytes and dendritic cells. Since these receptors recognise β2- microglobulin, they are not specific for HLA-G and are also activated by classical HLA molecules. However, affinity for HLA-G is considerably greater [156, 157]. The KIR2DL4 is of greater relevance in the uterine environment and inhibits local NK cell activity and the greatest significance of HLA-G may be at the foetal-maternal interface [158]. Most of the significance of the HLA-G molecule in transplantation and cancer immunology comes from its suppressive effects on T cell biology and this is mediated by the HLA-G1 and G5 isoforms [138](Table 1-1). Impaired sHLA-G secretion associated with reduced IL-10 levels has been reported in patients with UC, unlike healthy subjects and patients with CD [159].

The effects on the peripheral T cell populations have received particular attention in recent years and two "classical" effects are recognised. Naturally occurring CD4+ or CD8+ T cells expressing surface HLA-G arise in the thymus and are seen in the peripheral circulation of normal individuals. They do not express CD25 or FoxP3 and do not secrete IL-10 or TGF-β but are hypoproliferative and hyporesponsive to stimulation. Therefore they have regulatory functions but are distinct from T_R1 andT_h3 cells. The suppressive effects seem to be mediated by soluble factors such as HLA-G5 and are partially abolished by specific antibodies [160].

Membrane bound or soluble HLA-G down-regulates the expression of CD4 and CD8 on allo-stimulated T cells. These CD3 (+) CD4 (low) and CD3 (+) CD8 (low) T-cell subsets are Foxp3-negative suppressor T cells that secrete IL-10. Patients with high HLA-G plasma concentrations have higher numbers of these cells and after transplantation, had better graft survival compared with low HLA-G patients. These cells are similar to T_R1 cells, are low producers of CD4 or CD8 and have potent immunosuppressive properties [161]. When naïve T cells are sensitized with HLA-G5 through ILT-2 and ILT-4 receptors, they exert regulatory effects and become anergic [155].

In addition, HLA-G exerts effects on DC's through ILT4 receptors and on APC [162]. DCs are rendered tolerogenic as they do not up regulate co stimulatory molecules (CD80 and CD86) and MHC class II and instead, stimulate the expansion of CD4+CD25+CTLA4- and IL-10 producing CD8+CD28- Tregs [163]. A subset of DCs producing IL-10 (DC-10) is activated through an ILT4/HLA-G signaling pathway and induces antigen specific T_R1 cell differentiation. Suppressive effects through IL-10 can be expected [164].

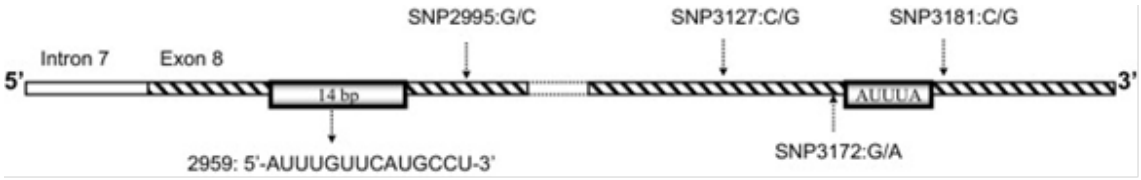
Although, many of these powerful suppressive effects have been examined in transplant biology they reveal effects that are significant across a variety of immune mediated inflammatory conditions.

1.2.1.8.2 Genetics

Over 40 HLA-G DNA alleles and sixteen HLA-G protein alleles have been reported. The most common allele is *HLAG*0101* and amino acid substitutions generate variants. A Thr31Ser creates the *HLAG*0103* allele, Leu 110 Iso creates the **0104* allele, Thr258Met at position creates the **0106* allele. Further subdivisions of the **0101* and the **0104* alleles are created by silent polymorphisms in exon 2 and 3. There are 2 null alleles: *HLA-G*0105N* where a deletion mutation in codon 130 in exon 3 creates a frameshift mutation and a premature stop at codon 171 and *G*0113N* where a point mutation at codon 54 in exon 2 creates a premature stop [165]. Subsequently, alternate isoforms are generated instead of the classical isoforms of HLA-G1 and HLA-G5 [166].

Altered expression of HLA-G has been associated with polymorphisms in the 3′ untranslated region (3′ UTR) and 5′ upstream regulatory region (5′ URR) of the gene. In the 5'URR, -G725C or -G725T seems to result in relatively low HLA-G expression and higher miscarriage rates [167] and this appears to be in LD with the 14 bp ins/del polymorphism in the 3'UTR [168].

A 14 base pair deletion/insertion polymorphism in the 3′ UTR region of exon 8 has been described and some transcripts associated with the insertion polymorphism undergo differential splicing to remove the first 92 base pairs of Exon 8 [169]. These shorter transcripts are stable, but the majority are not and hence, low sHLA-G levels are noted [169].



Reproduced from Larsen et al [165]

Figure 1-6 A schematic diagram of the 3'UTR demonstrating the recognized polymorphisms in this area

Although other polymorphisms in the coding region of the HLA-G gene have been described [170-173], the 14bp ins/del polymorphism has been extensively studied and the insertion is associated with recurrent spontaneous abortion, miscarriages, sarcoidosis and pemphigus vulgaris [139, 174-176]. Furthermore, strong LD exists within SNPs in the promoter region and the 14bp polymorphism [177]. Some have suggested that the HLA-G*010101 with the 14bp del allele is associated with higher levels of sHLA-G expression when compared with the *HLA-G *010102* haplotype with the 14bp ins allele but this effect has not been consistently observed [178] (Figure 1-6).

There is significant LD between *HLA-DR3* and *HLA-G*010102* in control populations and those affected by recurrent spontaneous abortions. For all four HLA loci studied, the alleles in the haplotype *HLA-DRB1*03.DQA1*05.DQB1*02.G*010102* were in clear LD [179]. LD has also been demonstrated for *HLA-DR3* and *HLA-G*010102* in individuals with RSA but not controls and *HLA-A1* and *HLA-G*010102* [173]. The HLA-A locus is close to the HLA-G locus on chromosome 6 and the *HLA1-B8-DR3* haplotype has been associated with colonic CD [58]. The *G*010102* allele includes the 14-bp sequence polymorphism in the 3' UTR of the gene and the 14bp ins/ins genotype was seen more frequently in CD patients who required ileo-caecal resection [177]. The haplotypes with the strongest LD with *HLA-G*010102* are not amongst those best associated with susceptibility or disease severity in IBD. Impaired sHLA-G expression has been reported in patients with UC, as compared to healthy subjects and those with CD. This difference was related to a reduction in IL-10 secretion in UC but not in CD [159]. HLA-G and IL-10 may be closely interrelated [180].

1.2.1.8.3 IL-10

IL-10 is an anti inflammatory cytokine and is produced by monocytes, macrophages and T_{reg} cells. It can induce T-cell anergy, inhibit MHC class I expression and the synthesis of pro-inflammatory cytokines by activated macrophages [181]. There is a compensatory increase of IL-10 in patients with CD and a more direct increase in patients with UC [182]. A relative deficiency of IL-10 in patients with UC could contribute to persistence of the inflammatory state [183] and IL-10 deficient mice develop spontaneous intestinal inflammation reminiscent of IBD [184]. These observations led to controlled trials of recombinant IL-10 for refractory CD but the results were disappointing. Recent genome wide association studies (GWAS) in adult [76] and paediatric [185] IBD cohorts have shown an influence of genetic variation in the IL-10 promoter region on susceptibility to IBD. IL-10 is clearly pivotal as a regulator of mucosal inflammation in IBD. However, the precise role awaits clarification. Expression levels are influenced by genetic variation in the promoter haplotype [186]. In normal individuals, the expression of IL-10 by stimulated PBMCs is influenced by three polymorphisms in the IL-10 promoter (G/A at -1082, T/C at -819 and A/C at -592). Three haplotypes are described of which GCC/GCC is a high producer, GCC/ATA and GCC/ACC are intermediate producers and ATA/ATA, ATA/ACC and ACC/ACC are low producers of IL-10 [187, 188]. Although initial trials studying the effect of systemic injection of recombinant IL-10 in IBD were only modestly effective in individuals with active CD [189, 190], more recent trials involving local delivery of IL-10 by lactobacilli in mouse models [191] and transgenic bacteria in humans have rekindled interest [192].

1.2.2 The clinical aspects of Inflammatory Bowel Disease

Whilst it is not always possible to successfully distinguish between the CD and UC, the distinction is often made on the basis of a discontinuous involvement of any part of the intestine from the mouth to the anus in CD as opposed to uninterrupted colonic inflammation, extending proximally from the rectum of varying extent in UC. CD is defined as a transmural intestinal inflammation affecting any part of the bowel; but typically the ileum and the colon. In UC inflammation is restricted to the intestinal mucosa and affects contiguous segments of the large bowel from the anal verge.

1.2.2.1 Clinical Features

Ulcerative colitis:

The symptoms of UC are generally those of bloody diarrhoea and urgency to stool, a small significant minority however, with rectal disease can present with constipation and rectal bleeding. The onset is usually sub-acute but can vary. The rectum alone is involved in 50% of cases. Of the rest, abdominal pain, cramping and fever are frequent. Increased stool frequency and noctural urgency to stool, systemic symptoms such as anorexia and weight loss suggest severe or extensive disease. Acute severe UC remains one of the main causes of potential mortality in UC and requires hospitalisation for intensive therapy and possible surgical management.

Crohn's Disease:

The symptoms are influenced by the part of the bowel predominantly affected. A further sub-classification is often used dividing patients into non-stenosing, non-penetrating, stenosing or penetrating. Diarrhoea, abdominal pain and weight loss are the commonest symptoms

Table 1-2 The influence of Crohn's disease phenotype on presenting symptoms

Disease Location	Type	Symptom	
Small Intestine (30%)	Stenosing	Abdominal pain , subacute intermittent intestinal obstruction, weight loss	Ileo-colonic disease is common and can result in a combination of these.
	Penetrating (entero-enteric fistula or abscesses)	Abdominal pain, fever, anorexia, weight loss	
Colon (30%)	Crohn's colitis	Severe Diarrhoea, rectal sparing	
Perianal	Penetrating	Fistulae (entero cutaneous, recto vaginal, colo vesical etc.) and perianal abscesses with perianal pain and fever.	

Classification of Crohn's Disease

The classification schemes have continued to evolve as we have seen earlier proposals on Phenotypic descriptions based on an anatomical location, give way to more modern concepts, based on disease behaviour and progression over time [193].

Table 1-3 A comparison of the Vienna and the Montreal Classification of Crohn's Disease

	Vienna Classification	Montreal Classification
Age at diagnosis	A1, less than 40 years	A1 < 17 Years
	A2, more than 40 years	A2 17 - 40 years
		A3 > 40 years
Location	L1, terminal ileum	L1, terminal ileum
	L2, colon	L2, colon
	L3, ileocolon	L3, ileocolon
	L4, upper gastrointestinal	L4, isolated upper
Behaviour	B1, non stricturing non-penetrating	B1, non stricturing non-penetrating
	B2, stricturing	B2, stricturing
	B3, penetrating	B3, penetrating
		P Perianal

1.2.2.2 The Course of Crohn's disease

The disease course is intermittent, remitting and progressive. Although some of the data demonstrating this comes from a time when surgery was the predominant therapy for severe CD, it is illuminating. At diagnosis, 35 % have pure ileitis, 36% have isolated colitis and 29% have ileocolitis [194]. Disease distribution usually remained stable and this distribution can provide an estimate of prognosis. An early onset of disease and ileal involvement predicted a penetrating course [195], and late-onset of disease was often limited to the colon [196]. A small number (1%) of patients have a continuously active course, 10% experience prolonged clinical remission and 50% with active disease may remain in remission for up to a year within three years of diagnosis[197].

A number of population-based studies have confirmed that disease course, over a period of 40 to 50 years, is characterised by an evolution in the nature of the inflammatory changes. The progress is one from an inflammatory (non-stricturing, non-penetrating type) to either of the two complicated types and was first noted in 2001 [198]. Most patients (usually around 60%) progress and a significant minority have established complicated disease at diagnosis (30%). Since then other reports have confirmed this and this has been the subject of a comprehensive review [196].

The landmark IBSEN population based study from Norway of 200 patients with CD follow-up over five years, demonstrated that the previously recognized progression of disease from one of the relatively mild non stricturing non-penetrating state to more complicated disease was not influenced by tertiary hospital referral bias [199]. However, the data does demonstrate the considerable bias towards a greater severity of the disease state that is evident at the referral centres.

Early Crohn's Disease

The availability of powerful, efficacious treatments in the secondary prevention of disease complications in "at risk" individuals mandates the description and the subsequent diagnosis of an early stage of disease. This is less easy to apply in CD particularly because of the frequent delay in diagnosis that many patients experience. Indeed, some authors suggest that 32% of individuals

may demonstrate evidence of established complications of disease at diagnosis. This includes stricturing or fistulisation [200]. It is also recognised that the disease often moves from a non-stricturing non-penetrating phenotype to the more complicated presentation over time [195, 198].

Such data coupled with the recognition of a guarded postoperative prognosis demonstrate the rationale of investigating a secondary prevention model. Unfortunately, this approach is limited by the diagnostic difficulty of a subclinical or mildly symptomatic state. Recent proposals have suggested a diagnostic algorithm based on clinical features, C reactive protein, endoscopic appearances, stool tests of inflammation and an early freedom from the need for steroids or immunomodulatory medication [201]. These efforts are clearly preliminary but accumulating evidence on the role of early immunomodulatory therapy such as AZA and Infliximab (INFX) will provide an impetus to identify reliable diagnostic criterion.

1.2.3 Treatment of the Inflammatory Bowel Diseases

On the whole, treatment regimens are relatively standard, but variations in clinical response and individual drug tolerance usually mean that the therapeutic approaches are individualised. The therapeutic approach is one of initial treatment to induce a clinical remission (induction phase) and then a subsequent maintenance phase to sustain this remission. Management in the traditional manner is based on the supposition that the least efficacious and least toxic medications are best used early when the disease may be relatively mild. The more efficacious and possibly more toxic drugs are therefore reserved for when the first choices fail; usually later in the disease course. This approach has undergone some modification with the recognition that certain disease groups are at greater risk of resistance to certain medications, aggressive disease behaviour and recourse to surgery. Small differences occur, but these are generally consistent across a number of practice guidelines form the US and Europe [202-204].

1.2.3.1 Ulcerative colitis

1.2.3.1.1 Induction Therapy

Initial therapy for patients with mild- moderate disease is 5- aminosalicylic acid. If this is unsuccessful, oral prednisolone in a dose of 40-60 mg a day, continued until clinical improvement occurs and then tapered over a period of time is beneficial and recommended. The 60 mg dose is more effective and causes more side-effects [203]. Anti TNF therapy, specifically INFX has been evaluated in moderate or severe UC and is effective in a dose of 5 mg per kilogram administered in the standard induction scheduled at 0, 2 and 6 weeks. In this moderately affected group, 22% of patients were able to achieve a steroid free remission at one year [205].

1.2.3.1.2 Maintenance treatment

Mesalamine suppositories [206] and enemas are effective in maintaining remission, when used regularly [207] or intermittently in a higher dose [208]. Steroids are not effective in the maintenance of remission [209] but the thiopurines, namely AZA and 6-MP; and INFX can be very effective. AZA and 6-MP are effective in maintaining remission in placebo-controlled maintenance trials [210]. Furthermore, it is generally well tolerated and could be considered in individuals who cannot tolerate mesalazine or sulphasalazine and subsequently require repeated doses of steroids [211]. INFX administered every eight weeks was effective in maintaining response in those in a remission at week 30 (30-50%) and week 54 (40%) [205].

1.2.3.2 Therapy of Crohn's Disease

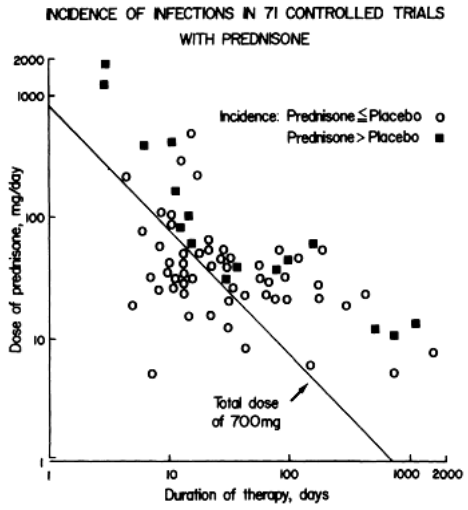
The severity of disease activity and location of disease are proposed as aids to rational therapy. A variety of guidelines are in existence that enshrines these principles [204, 212].

1.2.3.2.1 Induction of remission

Prednisolone is highly effective regardless of disease location with remission rates between 60 to 80% compared with placebo rates of 30 to 40% [213, 214]. Indeed, if the steroids are not tapered in the conventional fashion, remission rates of up to 92% are reported over seven weeks [215]. Unfortunately, 50% of all patients on corticosteroids will experience side-effects. Early effects

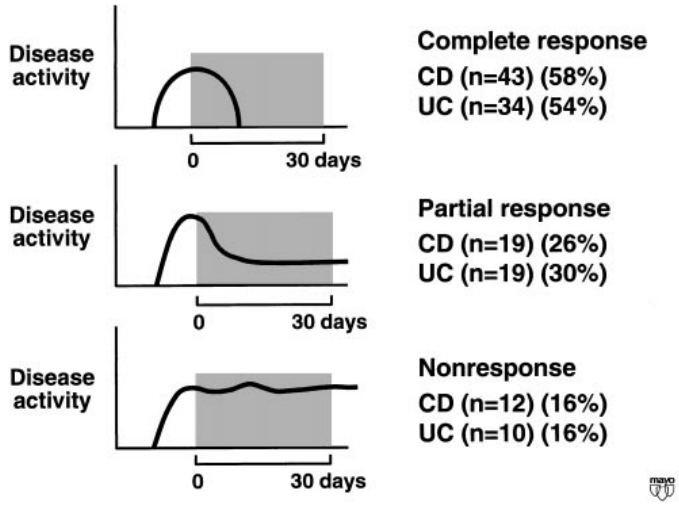
are predominantly cosmetic such as acne and moon facies. Subsequently, sleep and mood disturbances, and glucose intolerance may occur. A prolonged use of greater than 12 weeks could result in posterior subcapsular cataracts, osteoporosis and osteonecrosis of the femoral head, myopathy and increased susceptibility to infection [203]. Corticosteroid therapy predisposes to adrenal insufficiency, particularly in individuals who have received more than 30 mg per day of hydrocortisone of 7.5 mg per day of prednisolone for more than three weeks [216]. Symptomatic insufficiency is evident as postural hypotension, nausea, vomiting, hyperkalaemia and hyponatraemia. Steroid use increases risk of all infections (Figure 1-7) in particular, post operative infectious complications [217] This is particularly relevant in those who come to unplanned surgery.

Steroid usage is not uncommon and between 40 to 50% of all patients with CD will receive this treatment [218, 219]. Endoscopic mucosal healing is not usually evident [215] and should not guide decisions on treatment duration. Extending treatment duration, whilst reducing symptomatic relapse, does not increase time in remission after steroid withdraw [220]. Of those treated, 58% have a complete clinical remission at four weeks and 26% achieve a partial response. This “steroid free remission” is maintained in the minority (32%). 28% become steroid dependent and 40% require surgery [218] (Figure 1-8).



Above a total dose of 700mg the infection rates are significantly higher than placebo.

Figure 1-7 Graph demonstrating the increased risk of infections on steroid therapy [217]



Graph demonstrating that steroid use is initially effective but after a year is relatively inefficacious. Reproduced from Faubion et al. [218]

Figure 1-8 A demonstration of the Response to corticosteroids in patients with IBD

A variety of steroid tapering techniques are used around the world, with the expectation that most patients would be off steroid treatment in 8- 16 weeks [212]. Standard induction therapy would subsequently involve the commencement of immunomodulatory medication such as AZA, 6-MP or MTX and an attempt at weaning and maintaining a steroid free remission. However, patients who require steroids for remission induction are at risk of aggressive disease and up to 40 % of these patients will come to surgery within that year [218].

Steroid sparing Induction Regimen

In individuals who are resistant to or intolerant of steroid therapy, the anti TNF group of drugs offer an important alternative [221]. After the endoscopic confirmation of disease activity, treatment with this group of drugs should be started. Should these drugs prove inefficacious or if the patient experiences a loss of response, surgery should be considered [222]. The anti TNF therapy should be combined with AZA/6-MP as the combination is superior in maintaining the steroid free remission [223]

Perianal and fistulising disease

This is a particularly severe phenotype of CD and is at high risk of progressive complications over time. It affects the large majority of those with CD affecting the colon, increasing in frequency in rectal disease [224]. Although much of the evidence regarding the use of metronidazole and ciprofloxacin is from uncontrolled trials, it is recommended in most guidelines, commonly used and is often effective [225]. AZA/6-MP are used as second line treatment of CD fistula and a meta-analysis favoured fistula healing [226]. When these two treatments fail, INFX should be used. It should be continued to maintain the response that is seen in 36% of those treated [227, 228]. Therapy is often combined with surgical drainage of perianal abscesses and the use of setons [229]. Newer guidelines suggest that INFX and surgical therapy should be used early, perhaps as first line therapy as the disease course is adverse and is not adequately influenced by alternative regimen [230].

1.2.3.2.2 Maintenance of remission

The long-term maintenance of a clinical remission is beneficial and reduces the risk of hospitalisation and surgery [231]. While corticosteroids are not intended to be maintenance therapy, it is likely that some individuals will find themselves dependent on steroid treatment. Smokers and those with colonic disease appeared to be at greater risk [232]. Patients needing corticosteroids for longer than 3 months are at particular risk of osteoporotic complications and recommendations exist for bone mineral density screening and replacement therapy with calcium, Vitamin D and bisphosphonates [203]. Neither sulphasalazine [213, 219] nor mesalazine [233] maintain a steroid induced remission. AZA and 6-MP are effective in maintaining steroid free remission [234], induced by steroid therapy for up to four years [235]. Weekly MTX in a dose of 25 mg per week is steroid sparing and 15 mg intramuscular maintains a MTX induced remission [236]. Scheduled, eight weekly infusion of anti TNF therapy (Infliximab) maintains remission in luminal and fistulising CD. Adalumimab and Certoluzimab are most likely equally effective alternatives [222].

1.2.3.2.3 Mucosal healing and top-down therapy

It is now accepted that healing of colonic or ileal mucosa on therapy imparts a significant advantage to patients. In UC steroids, mesalamine, immunomodulators and biologics can induce mucosal healing but steroids cannot maintain the healed mucosa. In CD, a similar observation is true except that meslamine is ineffective. Mucosal healing in CD treated with AZA/6-MP varies from 15% after 26 weeks [223] to 58% at 1 year [237]. Although there are no randomised controlled trials to examine these as a primary endpoint, it appears that the effect takes time but is achievable.

Much of the data supporting the benefits of mucosal healing come from sub-group analysis of studies of INFX use, however there is a valuable population cohort study of the prebiological era that demonstrates that mucosal healing reduces the need for surgery in both UC and CD with benefits seen for upto 10 years. Healing at 1 year in this study identifies a low risk group [238]. There is obviously no study of mucosal healing in biologicals of this magnitude as the experience has not built up as yet.

MTX is also capable of mucosal healing in CD in up to 40-50% of individuals [239]. Although the studies supporting these observations are substantially smaller, they provide valuable support for the role of immunomodulation in IBD. It is important to highlight the further impact of the role that MTX currently occupies; namely that of second line immunomodulation after AZA failure or intolerance. These rates of mucosal healing under these limitations are impressive indeed.

The recognition that treatment efficacy varies with time, with both INFX and AZA demonstrating greater response rates, when used earlier has led to criticism of traditional treatment regimen. The knowledge that up to 30% of patients have a benign course of disease, not requiring immunomodulation [240], supports a need for predictors of a severe disease course.

1.3 The immunomodulators

1.3.1 Azathioprine

Much like other drugs used in the treatment of autoimmune conditions, AZA was developed to treat childhood leukaemia. A decade after the discovery by Hitchings and Elion [241], the first report was published on the use of 6-MP in CD [242] and another 10 years on, the first clinical trial in IBD was reported [243]. These drugs are now standard therapy in UC and CD.

1.3.1.1 Pharmacokinetics

Mercaptopurine contributes to approximately 50% of the molecular weight of azathioprine and 90% of azathioprine is converted to this form. The oral bioavailability of azathioprine is highly variable and ranges from 25%- 80% [244]. 6-MP has a short plasma half life of between one to two hours [245].

1.3.1.2 Metabolism

AZA is a pro-drug of the thiopurines analogue, 6-MP. Neither has intrinsic activity, and need to undergo enzymatic metabolism to generate active molecules. AZA is undetectable in the bloodstream since methyl-nitrothioimidazole is rapidly cleaved probably within red blood cells [246] and hepatocytes [247]. This is predominantly enzymatic in the liver, mediated by glutathione S-transferase (GST). In the erythrocyte, it is non enzymatic and may be mediated by compounds such as glutathione or cysteine. The relative contribution of these individual sites is unknown but hepatic metabolism may be very relevant.

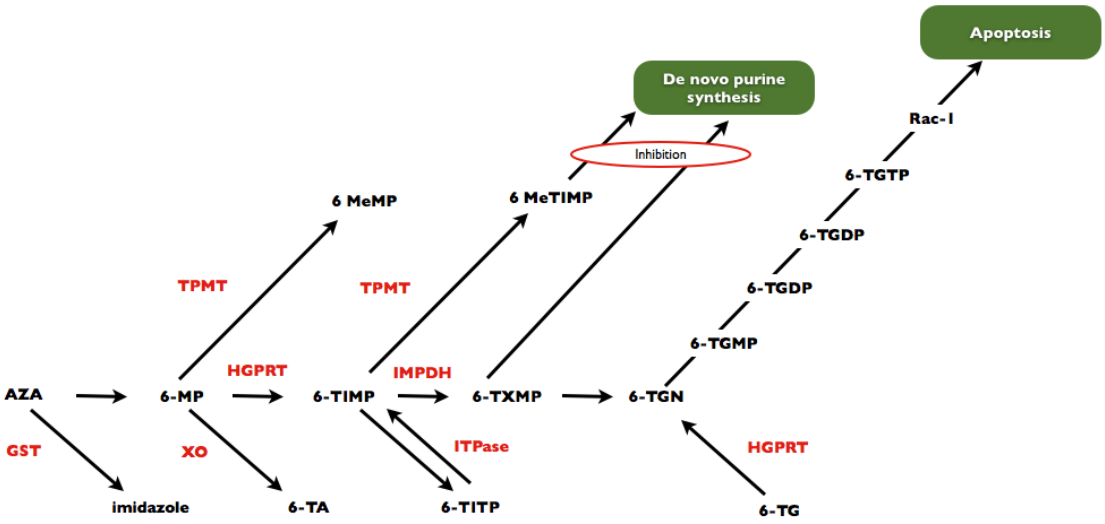
Intracellularly, 6-MP is faced with three competing enzyme pathways (Figure 1-9). These are very similar to those used by endogenous purines [248]. 6-MP is metabolised by xanthine oxidase (XO) to 6-thiouric acid (6-TU); an inactive metabolite identifiable in plasma and urine [249]. The active compounds, 6-thiogunaine nucleotides (6-TGN) are formed from 6-MP by the action of hypoxanthine guanine phosphoribosyltransferase (HGPRT), inosine 5-monophosphate dehydrogenase (IMPDH) and guanidine 5- monophosphate synthetase (GMPS). One of the intermediaries in this pathway, thioinosine monophosphate (tIMP) is a substrate for TPMT,

leading to methylated thioIMP (MeTIMP) and also 6-thioxanthine monophosphate (6-TXMP) [250] which inhibit de novo purine synthesis [251]. The predominant inactivation pathway of thiopurines in haematopoietic cells is through the methylation of 6-MP by thiopurine-s-methyl transferase (TPMT) generating 6-methylmercaptopurine (6-MMP). TPMT is affected by genetic polymorphism and can manifest a 50 fold variation in enzyme activity between individuals [252]. Thus, if this inherited deficiency is present in an individual treated with 6-MP, they are likely to generate excessive concentrations of the active compound 6-TGN with a high likelihood of toxicity. Conversely, augmented enzymatic activity would lead to attenuated 6-TGN generation and a compromise in therapeutic efficacy.

1.3.1.3 Mechanism of action

Although, thiopurines have found widespread application in a variety of conditions, the exact mechanism of the immunosuppressive effect remains unclear. There are a number of contributory influences. These are as follows:

1. 6-TGN may be incorporated into DNA and RNA resulting in inhibition of nucleotide protein synthesis and ultimately, lymphocyte proliferation [253].
2. 6-TGN accumulates in lymphocytes, and when T-cells are activated, it blocks expression of TRAIL, TNFR57 and α4 integrin and subsequent inflammation [254]. This may be relevant in lamina propria T-cells in IBD.
3. AZA and its metabolites control T-cell apoptosis by modulation of Rac-1 activation converting a stimulatory signal into an apoptotic signal. 6-thio-GTP binds to Rac-1 instead of GTP and genes such as MAP-Kinase, NF-κB, and bcl-xl are suppressed [255].
4. 6-MP impairs *in vitro* differentiation of dendritic cells (DC) and has an inhibitory effect during DC activation processes inducing a tolerogenic phenotype. 6-MP significantly reduces IL-23 production and CCR7 expression and augments IL-10 [256].



AZA: azathioprine, 6-MP: 6-mercaptopurine, GST: Glutathione S Transferase, TPMT:Thiopurine methyl transferase, 6-TA: 6-Thiouric acid, XO:Xanthine Oxidase, 6-TIMP:6-thio ionosine monophosphate, 6-TXMP:6-thioxanthine monophosphate, 6-TGMP:6-thioguanine monophosphate, 6-TGDP: 6-thioguanine diphosphate, 6-TGTP: 6-thioguanine triphosphate, 6-TITP: 6-thi inosine triphosphate, 6-MeMP: 6-methyl mercaptopurine, xo: Xanthine Oxidase, IMPDH: inosine monophosphate dehydrogenase, HGPRT: hypoxanthine phosphoribosyl transferase

Figure 1-9 Azathioprine metabolism and a proposed mechanism of action

1.3.1.4 Pharmacogenetics and Metabolite monitoring

1.3.1.4.1 TPMT

The pharmacogenetic influence of TPMT on AZA metabolism remains one of the great success stories in this field. Although, it is clinical application has been indirect with TPMT phenotype rather than genotype finding widespread use, it is clear that the findings of the genetic influence was crucial. A number of single nucleotide polymorphisms (SNPs) for TPMT have been identified. The nomenclature is defined and follows clear criterion for clarity. The gene and the allele are separated by an asterisk and the alleles are represented as Arabic numerals. Further nucleotide changes are assigned letters that follow the Arabic numeral. The nucleotide numbering starts at A in the ATG start codon and are designated +1 [482]. Most of these are rare other than the **3A/C* alleles. The nucleotide changes for *TPMT*3A* are 460G>A and 719A>G, *TPMT*3B* is 460G>A and *TPMT*3C* is 719A>G [482]. Nearly 90% of Caucasian people have a wild type genotype (**1/*1*) and subsequently normal TPMT enzymatic activity. 10% of this population are heterozygous (**1/*3*) and have reduced TPMT activity, which may be as low as 50% of wild type activity. One in 300 individuals are homozygous mutants or compound heterozygotes *(*3/*3)* and have no TPMT activity [257]. This usually correlates with enzyme assays performed by high performance liquid chromatography (HPLC) and the combination of the two identifies accurately the one in 300 individuals at risk of profound haematological toxicity when treated with thiopurines in the standard dose [258]. Heterozygous individuals with intermediate TPMT activity also have a high risk of haematological side-effects when treated with standard doses of AZA [259]. Myelosuppression on AZA therapy occurs in up to 5% of patients [260] and this is thought to be dose related effect due to a elevated 6-TGN. Gastrointestinal side effects were not influenced by TPMT activity and the majority of individuals with haematological toxicity do not have impaired TPMT [261]. TPMT heterozygosity can lead to GI intolerance if individuals are exposed to full dose AZA (2- 2.5 mg/kg). This often leads to withdraw from therapy [262, 263] and avoids haematological toxicity that may have occurred a few months later [264].

1.3.1.4.2 ITPA

Since the majority of drug side effects to AZA and 6-MP are not attributable to variation in TPMT activity, other genetic influences have been explored. The enzyme, Inosine triphosphate pyrophosphatase (ITPA), mediates a futile cycle in purine metabolism [265]. In an individual

with low ITPA activity, exposure to AZA leads to accumulation of the methylated thio-ITP which may be toxic [266]. The ITPA 94A>C mutation has been implicated in causing rash, flu like side effects and pancreatitis and haematological toxicity in some cohorts but not in others. The clinical application is therefore currently uncertain.

1.3.1.4.3 Xanthine oxidase/dehydrogenase and Aldehyde oxidase

A recent report has suggested that a polymorphism in XDH appeared protective against side effects on AZA or 6-MP and that an additional polymorphism in the AOX gene reduces response to AZA therapy [267]. These findings are preliminary and require replication.

1.3.1.4.4 IMPDH and HGPRT

The central role of IMPDH makes it a likely candidate for inter-individual variation in activity and hence pharmacogenetic influence. However this has not proved to be the case to date [268]. HGPRT deficiency or genetic variation reducing enzymatic activity would also be predicted to have pharmacogenetic influence. However, the deficient state is not consistent with significant survival and is therefore not clinically relevant [269].

1.3.1.4.5 Glutathione-S-transferase (GST)

Glutathione-S-transferase (GST) activity also varies according to genetic polymorphism. Polymorphism in GST-M1 has been shown to increase risk of lymphopenia in patients on thiopurines [270] rather than the expected hepatotoxicity and pancreatitis.

1.3.1.5 Adverse Effects

As previously described, adverse effects rates range from 5-13 % and two categories are described.

1. Idiosyncratic (dose independent)

Up to 6.5% of individuals have idiosyncratic side effects including nausea, fever, rash, flu like illness, pancreatitis, hepatitis and arthralgia. These symptoms often occur within 4 weeks of starting therapy and recur on re-challenge. In some cases, particularly nausea on AZA, side

effects can be overcome by switching to 6-MP suggesting involvement of the nitro-imidazole moiety [271]. Switching to 6-MP is not advisable in those individuals who have experienced acute pancreatitis.

2. Dose dependent

Myelotoxicity is dose dependent and occurs in 1.4% – 5% of patients [260, 272]. It can be caused by TPMT deficiency, TGN excess, viral infections, co medication such as 5-ASA, aspirin, allopurinol, frusemide, MTX and potentially INFX. It can occur at any time from the start of treatment up to 11 years and [210] and is managed by dose reduction or withdrawal of therapy. Hepatotoxicity is well known but relatively infrequent occurring in around 5% of patients [273]. It is usually detected as an elevation in liver enzymes and the patients are often symptomatic with fatigue, nausea and headaches. Withdrawal or dose reduction leads to resolution of symptoms [272]. Other more unusual conditions due to veno occlusive disorders may be seen.

1.3.1.6 Clinical applications of AZA/6-MP in IBD

The hospitalization rates for patients with CD have declined [274] and may imply an improvement in the management of the chronic inflammatory state by immunomodulatory medication. It is recognized that 5 ASA medications is inefficacious in CD, and that steroids cause cumulative problems. It may be reasonable to attribute this is decline in hospitalisation rates to increases in the clinical use of AZA or 6-MP.

AZA and probably the equivalent 6-MP are effective in the induction of a clinical remission in individuals with active CD. It is effective in 54% of treated patients as opposed to a 34% efficacy for placebo. This gives an OR (odds ratio) of 2.4 (95% CI 1.6- 3.6) and a number needed to treat (NNT) of 5 for clinical efficacy. However, the time to effect may be prolonged and use as the sole agent in therapy is not easy. Steroids are often required for symptom control and improve the OR for response to 2.6 (95%CI 1.6 – 4). A steroid sparing effect is prominent and the OR of achieving a reduction in steroid dosage but not necessarily complete withdrawal is 3.6 (95% CI 2-6.4) [203, 204].

AZA is effective in maintaining a remission that has been induced with or without steroids. The OR is 2.3 (95% CI 1.55 to 3.5) with an NNT of 6. 6-MP has a slightly higher OR (3.32, CI 1.4 to

7.8) on the back of a smaller group of patients. A dose response and a steroid sparing effect are seen. The OR for response increases from 1.2 to 4.13 as the dose increases from 1mg/kg to 2.5 mg/kg per day. The risk of side effects increases on treatment when compared with placebo [203,204].

The evidence for AZA/6-MP in the maintenance of a remission in UC has been more uncertain but a recent meta analysis [210] and "second look" [275] have both found that it is effective in this setting and it is recommended in practice guidelines for the maintenance of a remission[203, 276].

Duration of treatment with thiopurines in IBD

An observational study by the GETAID group in patients with CD demonstrated that after four years of therapy, the longer the duration of remission on AZA therapy, the lower the risk of relapse [277]. A report from the Oxford group on a 30 year experience of the use of AZA demonstrated that prolonged use was safe and effective in the long term with 60% of individuals maintaining a remission up to 5 years [278]. However on subsequent investigation, in a placebo-controlled trial it was evident that prolonged AZA administration maintained a greater proportion of patients in remission [235]. Similar findings were reported by the Oxford Group [278] and this, associated with the recognition of a low risk of cumulative side effect rates on prolonged treatment and, it is now conventional for patients with CD to maintain long-term therapy.

The mechanism of action has always lent itself to anxieties about the risk of malignancy on long term therapy. As described, the details of the mechanisms involved are unclear but all the proposed options could increase risk of carcinogenesis. There have been concerns about risk of cervical cancer, colorectal carcinoma and lymphoma. On balance it appears that the lymphoma/leukaemia risk is the most significant but taken in light of the low risk of one extra lymphoma in 300- 1400 years of treatment, it seems reasonable to recommend continued therapy [279]. It may be beneficial to monitor metabolite levels in those on established therapy with a view to minimizing the risk of persistently supra-therapeutic TGN levels and subsequent DNA instability. This approach has not been evaluated and will be difficult to examine in light of the low risk of malignancies attributable to AZA/6-MP therapy.

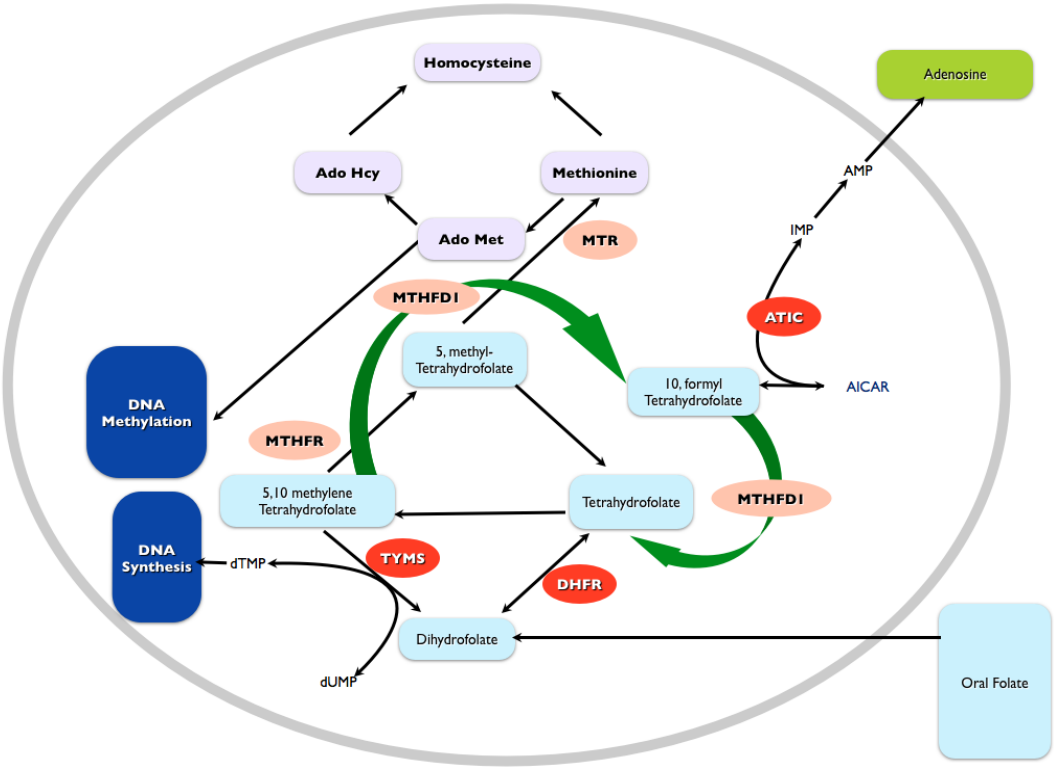
1.3.2 Methotrexate

1.3.2.1 The Folate pathway

Folates are water-soluble B vitamins required for normal cell growth and metabolism. They play an essential role in 1-carbon metabolism and the subsequent biosynthesis of purines, thymidylate and other amino acids [280]. Polyglutamated dietary folates undergo deconjugation by folyl polyglutamate hydrolase in the intestine cell wall to the monoglutamate form and are then transported to the liver.

Cellular transport is mediated by the bi-directional, high-capacity pH transporter molecule; reduced folate carrier (RFC) [281, 282]. Within the cell, folylylpolyglutamate synthase (FGPS) mediates the sequential addition of glutamate residues onto the proximal glutamate of folic acid increasing the chain length. Increased polarity and cofactor affinity results [283] and keeps the molecule within the cell. Gammaglutamate hydrolase (GGH) mediates the removal of polyglutamate groups. This balance of polyglutamation and deglutamation maintains high intracellular folate levels; 1-10 μmol compared to 10nMol/L in the plasma [284]. These folates are reduced to dihydrofolate (DHF) and then to tetrahydrofolate (THF) by dihydrofolate reductase (DHFR). The addition of a hydroxy methyl group forms 5-10, methylene tetrahydrofolate (5, 10-CH2-THF). Methylene tetrahydrofolate reductase (MTHFR) is a critical enzyme and mediates the irreversible donation of a methyl group from 5, 10-CH2-THF creating all the circulating folate or 5-methyl THF. A methyl group is donated towards the remethylation of homocysteine from methionine. This is the precursor of S-adenosyl methionine (SAM), the universal methyl donor important in many reactions including DNA methylation [285]. In effect, MTHFR diverts DNA synthetic activity towards DNA methylation. 5, 10-CH2-THF is also a cofactor for the enzyme thymidylate synthase (TS) and transfers a one carbon unit from dUMP (deoxyuridine monophosphate) to dTMP (thymidine monophosphate) [286].

Reduced folate derivatives including 10-formyl-THF provide one carbon units for de novo purines synthesis. Glycinamide ribonucleotide transformylase (GARTF) contributes to the imidazole ring of purines and 5-aminoimidazole-4- carboxamide ribonucleotide transformylase (AICARTF or ATIC) provides the purine inosine 5’-monophosphate (IMP).



dTMP: deoxy thymidine monophosphate, dUMP: deoxy uridine monophosphate, TYMS: thymidylate synthase, DHFR: dihydrofolate reductase, ATIC : 5-aminoimidazole-4-carboxamide ribonucleotide transformylase, MTR: methionine synthase, MTHFD1: methylene tetrahydrofolate dehydrogenase, AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide, IMP: inosine monophosphate, AMP: adenosine monophosphate, AdoMet : S- adenosyl methioninse/SAM, AdoHcy : S adenosyl homocysteine /SAH

Enzymes in red are inhibited by Methotrexate. Folate pathway enzymes not affected by MTX are coloured pink. Folates are coloured blue and the methionine /homocysteine pathway is coloured purple.

Figure 1-10 The folate pathway

1.3.2.2 A history of Methotrexate

Following the discovery of antibiotics, a growth factor essential to the bacterium Lactobacillus casei was isolated and identified as folic acid. When administered to patients with leukemia, a symptomatic deterioration was observed [283]. The pursuit of a hypothesis that a folate antagonist would be a therapeutic option led to the synthesis of aminopterin. This was created by a substitution of the hydroxyl group at position 4 of a pteridine ring of folic acid for an amino [287]. This key change allows for the tight binding and inhibition of DHFR although this was not recognised initially.

Aminopterin's toxicity led to a search for a superior alternative. Methotrexate (MTX), a 4-amino 10-methyl substituted analogue, initially used in childhood acute lymphoblastic leukaemia, went on to find a curative application in lymphoma and choriocarcinoma.

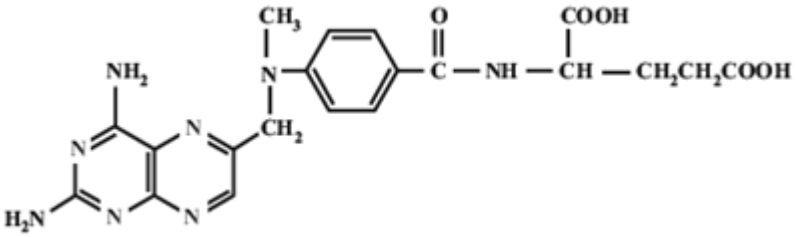


Figure 1-11 Chemical structure of Methotrexate

Interesting observations on the resistance of acute myeloid leukaemia to methotrexate and the sensitivity of acute lymphoblastic leukaemia to the same drug provided impetus to the study of resistance mechanisms. This intrinsic difference appeared to be due to defective polyglutamation in Acute myeloid leukaemia (AML).

1.3.2.3 The mechanism of action of Methotrexate

After the development of MTX in 1948 by Farber et al, it quickly found clinical application in RA [288] albeit without any real understanding of mechanistic influences. Since then, considerable progress has been made. Aldehyde oxidase (AOX) mediates the hepatic oxidation of MTX to its primary metabolite 7- hydroxy MTX (7-OH MTX) [289]. 7 -OH MTX is the dominant anti folate 42 hours after administration. Like MTX, and with a similar affinity, SLC19A1 allows the cellular influx of 7-OH MTX [290]. Inside tumour cells, 7-OH MTX is a good substrate for FGPS allowing a high level of polyglutamation [291, 292]. However, the resulting 7-OH derivatives are inferior DHFR inhibitors diminishing the cytotoxic efficacy of MTX [293].

MTX competes with folates for active transport by the low capacity transporter [294] SLC19A1 and the alternative folate transporters [295] into the cell. Efflux is mediated by ATP-binding cassette (ABCC1-5, ABCG2) transporters and breast cancer receptor protein (BCRP) [296, 297]. Intracellularly, MTX tightly binds DHFR and subsequently dihydrofolate (DHF) accumulates. FGPS mediated polyglutamation increases the intracellular half life of MTX [298]. GGH sequentially removes the polyglutamate allowing efflux of MTX from the cell. When polyglutamates accumulate, DHFR, TS, AICARTF and ADA [299-301] inhibition is evident.

DHFR is a key enzyme in the folate pathway and generates 5–methyl-THF. This donates a methyl group and allows for the conversion of homocysteine to methionine. Methionine is required for the synthesis of SAM, which is further converted to s-adenosyl homocystine (SAH) [302]. In a normal cell, DHFR activity is higher than the activity of TS. Therefore, DHF is present at low levels. Consequently, a small amount of DHFR activity fulfils the cellular need and DHFR inhibition leads to a profound failure of the regeneration of THF from the DHF.

MTX affects TS in two ways. There is a failure of downstream pathways dependent on 5-10-methylene tetrahydrofolate as a substrate; particularly TS catalysed dTMP formation from dUMP [303, 304]. MTX also directly inhibits TS. The effects are profound with partial depletion in the TS substrate, 5-10- methylene tetrahydrofolate [305], deoxythymidylate monophosphate (dTMP) depletion, mis-incorporation of the uracil cell base into DNA, chromosomal damage and

subsequent cell death. Also known as thimydineless death, this term emphasises the importance of the effect on TS [306]

Methotrexate does not directly affect MTHFR but variant enzymatic activity along with the MTX induced folate pool depletion, leads to a profound loss of 5-methyl THF and subsequent vitamin B12 dependent remethylation of homocysteine to methionine [307]. The downstream loss of SAM is manifest in the defective methylation of nucleotides in DNA, RNA and proteins [308].

However, some clinical experience and controlled randomised studies in RA demonstrate that oral folate supplementation does not modify response to low dose MTX therapy but instead reduces complications attributable to the antiproliferative effects of MTX and hepatotoxicity [309]. This suggests that folate independent mechanisms are at play. MTX also mediates important effects on purine metabolism. The polyglutamated form competitively inhibits AICARTASE/ATIC [310] and AICAR accumulates [311]. AICAR riboside inhibits the catalytic activity of adenosine deaminase (ADA) and AMP deaminase [301, 310]. ADA is also affected by competitive inhibition by MTX [312]. The resulting accumulation of adenine and adenosine nucleotides leads to extracellular release and subsequent conversion by extra cellular 5' nucleotidase to adenosine [313]. Adenosine is difficult to investigate or measure due to a short half-life but elegant experiments have demonstrated that adenosine receptor antagonists and adenosine inactivators such as ADA reversed the effects of MTX on leucocyte accumulation in an animal model [314, 315]. Adenosine inhibitors such as theophylline and caffeine reverse the development of adjuvant arthritis in an animal model [316] and caffeine consumption reduces the efficacy of methotrexate in humans with RA [317]. In animal experiments, the anti-inflammatory effects of MTX appear to be mediated by adenosine receptors A_{2A} and A_3 [318]. Some conflicting evidence has been provided where in an animal model, the anti inflammatory effects of MTX were not abolished by adenosine receptor antagonists but instead by folic acid supplementation [319]. The doses of MTX used were considerably higher than in earlier experiments and is an illustration of the differences between low dose and high dose MTX therapy.

The effect of MTX on Cytokines

The normal cytokine production following T-cell activation in whole blood cultures is inhibited by methotrexate. IL-4, IL-6, IL13, TNF- α, interferon-gamma (IFN-γ) and GM-CSF are reduced.

The addition of folic acid, hypoxanthine and thymidine, or adenosine reversed the inhibitory effects of methotrexate suggesting that the cytokine inhibition followed *de novo* synthesis of purines and pyrimidines. Furthermore, cytokine production by monocytes is modest [312, 320-323]. MTX did not affect LPS induced cytokine IL-1β or TNF-α alpha release in monocytes. [324]. A sex differential on the anti-inflammatory effect of MTX on monocytic cells has been suggested, which may explain an observed increase efficacy of MTX in male patients [325].

MTX affects the cytokines via the NF-κB signalling pathway. TNF alpha binding activates this pathway, which subsequently upregulates a variety of genes. MTX inhibits IκBα. Consequently NF-κB stays in the cytoplasm and the signalling pathway is not activated [326].

Other possible mechanisms of therapeutic efficacy of low dose Methotrexate

Another proposed mechanism is that MTX inhibits the generation of polyamines which in turn generate lymphotoxic NH_3 and H_2O_2. This occurs as a consequence of the previously described decrease in SAM which in turn is required for the methylation and synthesis of spermine and spermidine as reviewed by Cronstein [327].

Other mechanisms have been explored and some evidence suggests that the effects of MTX *in vivo* may be due to T-cell apoptosis, modified cytokine production and reduced T cell proliferation. A role for intracellular reactive oxygen species (ROS) levels, inhibition of pyrimidine pathway enzymes and increasing CD95 sensitivity of CD 45+RO cells (activated T-cells) has been demonstrated. The correlation between apoptosis and ROS generation was shown in lymphocytic cell lines but not in monocytes. This may be due to a higher level of antioxidants in monocytes preventing ROS induced apoptosis. MTX induces ROS in a time and concentration dependent manner [328, 329].

Further evidence for methotrexate induced apoptosis is seen on prolonged incubation of activated T-cells with MTX leading to a CD95 sensitive state. CD95 is a member of the death receptor family. This increased sensitivity was seen with CD 45+ plus RO cells [330].

Methotrexate may be more beneficial in active RA, rather than exhibiting a general antiproliferative state in RA. This has been inferred from the reduction of the CD4+ CD28 + cell

population and the CD4+CD28- population in active RA. In non-active RA, these cell populations were unaffected [329]

1.3.2.4 Methotrexate Pharmacokinetics

The dosage used is important and the route of administration may be too, although this is less certain. A distinct lack of consensus about the optimal mode of administration in IBD has contributed to the uncertainty regarding the place of MTX in the therapeutic strategy for CD [331]. Variability in bioavailability following oral administration (80-25%) has led to recommendations for parenteral administration. However, food does not modify absorption [332] and subcutaneous dosing is comparable to IM administration with a bioavailability of more than 80% [333]. The drug is absorbed from the proximal intestine by relatively specific transport mechanisms and is not reduced in patients with small bowel CD. Bioavailability of the drug has been reported to drop on dose escalation [334], but the result is insignificant in the doses used for IBD [335].

A report of the Edinburgh experience in the treatment of 39 CD patients where the excellent initial response to IM MTX (71%) was lost over 50 weeks with a probability of relapse of 78% appears to supports the preference for parenteral administration, but in fact there were a considerable number of steroid dependent patients in the oral conversion group. These individuals could be considered to be MTX treatment failures that were steroid dependent and relapsed when the steroids were tapered [336]. It could also be a result of the described effect of steroid use increasing intracellular polyglutamate levels [337] which then drop when the steroids are withdrawn. Furthermore, the impact of diminished adherence is difficult to estimate. There is considerable inter-individual variation in the intracellular levels of the polyglutamated form of MTX. These are proposed to correlate with clinical response to MTX in RA [338] but the variability makes clinical application difficult [339]. Notwithstanding this, the results of dosing studies on intracellular polyglutamation levels clearly demonstrate that higher levels are achieved with higher dosages [337] and possibly, parenteral dosing. The use of the parenteral route is recommended in practice guidelines but it is thought that a switch to oral therapy would be preferred by patients [222].

The predominant renal clearance of the drug means that patients with renal impairment are at risk for toxicity [340]. Owing to its significant intrahepatic recirculation and the persistence of a polyglutamate form, the drug can be slow to be cleared completely [341].

1.3.2.5 The use of MTX in the Inflammatory Bowel Diseases

Immunosuppressive medication is used in patients who are steroid responsive but dependent or those who are steroid refractory [204]. In CD, these are primarily the thiopurines (AZA/6-MP) and MTX and both are similar in efficacy and tolerability [342, 343]. However, clinical experience and the volume of trial evidence have made AZA/6-MP fist choice. MTX is an option for remission induction and maintenance of that remission in those resistant or intolerant to AZA/6-MP [203].

MTX use was first described in the treatment of patients with IBD in a small, uncontrolled study of 21 patients (14 with CD and 7 with UC). Almost all patients had either failed to respond to or tolerate AZA/6-MP and were steroid dependent. After an initial induction dose of 25mg IM for 12 weeks, a dose reduction to 15 mg orally was undertaken in responders. Sixteen patients (11/14 CD and 5/7 UC) responded to treatment with significant improvement in CDAI and UC activity indices and a reduction in steroid usage. 30 % were able to withdraw from steroids. Mucosal healing was noted in 4/14 CD (4 of the 11 responders, 36%) and 0/7 UC patients. Side effects were minor (transaminitis in 2, nausea in 2, minor leucopenia in 1 and minor pneumonitis in 1). Folate supplementation was not used. [344]

The much quoted study by Feagan et al reported in 1995 was a randomised, double-blind, placebo controlled, multicentre study of 141 patients with steroid dependent CD. Patients received weekly injections of MTX (25 mg/week IM) and a prednisolone taper from 20 mg/day was attempted. 37/94 (39%) in the treatment group as opposed to 9/47 (19%) in the placebo group were free of steroids and in a clinical remission as per CDAI ($p=0.026$, RR 1.95, CI= 1.09 – 3.48). Steroid use and mean CDAI was lower in the MTX group. Sixteen patients (16/94, 17%) in the MTX group withdrew because of side effects versus 1/47 (2%) in the placebo group. The reasons for withdrawal were liver function test (LFT) abnormalities in 7, nausea in 6, skin rash in 1, pneumonia in 1 and optic neuritis in 1. The incidence of minor side effects allowing continuing

treatment was not significantly different between the study group and placebo patients. Folate supplementation was not used [345].

Another randomized double-blind placebo-controlled trial of MTX in 33 patients with steroid dependent CD was reported a year later. One third of these had previously failed treatment with a thiopurine. Patients were given placebo or oral MTX 15 mg per week, adjusted up to 22.5 mg per week for up to 12 months in responders. 28 patients completed the study 13 patients received MTX and 15 received placebo. 46% of MTX treated patients relapsed, as compared to 80% of placebo treated patients. Due to the small numbers examined this did not reach statistical significance. There was a non significant trend towards increased number of significant side-effects in the MTX group (3/13 or 23%) as compared to placebo group (0/15 or 0%). Folate supplementation was not offered [346].

Feagan et al conducted another randomised, multicentre, placebo-controlled trial of 76 patients with chronic active CD, who had previously entered remission with 25 mg of methotrexate/week by IM injection for 16 weeks. Remission was defined as a withdrawal of steroids and the maintenance of a score of 150 or less on CDAI. 40 patients received MTX and 36 received placebo. The methotrexate group received 15 mg/week, IM for 40 weeks, no other treatments were permitted. At the end of the study, significantly more patients in the MTX group remained in remission (26/40 or 65% versus 14/36 or 39%). Steroid usage was less in the MTX group (28% versus 58%). Some of the patients who relapsed (n= 22) were re-induced with 25 mg/week MTX administered IM. Of these, 12/22 (55%) regained their remission and successfully withdrew from steroid treatment. Of those who did not receive methotrexate after relapse 2/14 (14%) were in remission at week 40. None of the patients in the methotrexate group had a severe adverse event. Symptoms attributable to active inflammatory bowel disease were more common in the placebo group (abdominal pain, fatigue, diarrhoea, joint pain). Nausea and vomiting, was more common in the methotrexate group 16/40 (40%). One patient in the MTX group withdrew from treatment because of nausea. No patients suffered significant haematological or pulmonary complications. Folate supplementation was not used [347].

In a double blinded, randomised study from Milan, 54 patients with steroid dependent CD were treated with IV Methotrexate 25 mg/week or 2 milligram/kg/day of AZA. A steroid taper was

attempted in all patients. After the first three months, IV methotrexate was changed to the oral formulation in the same dose. No significant difference was found between the two regimen to clinical response at three or six months (6 month response- methotrexate 56%, AZA 63%, p = 0.39). 3/27 (11%) patients treated with methotrexate (thrombocytopenia-1, leuco thrombocytopenia-1, hepatitis-1) and the same number in the AZA group (fever/headache-1, pancreatitis-1, hepatitis-1) withdrew from the trial due to adverse events. One patient in each group required dose reduction for control of leucopenia and impaired LFT. Toxicity such as nausea and vomiting, not requiring withdrawn from treatment were more commonly seen in the methotrexate group (44%) vs 7% in the AZA group. Folic acid was not administered [342].

Table 1-4 Effectiveness of Low Dose MTX in Induction (I) and Maintenance (M) of Remission in IBD from placebo controlled trials

Study	Disease	Clinical Response		p	OR	Ref
		MTX-Treated n1/N1	Control			
Feagan et al	CD	37/94	4/47	0.016	2.741	[345]
Oren et al	UC	14/30	18/37	0.872	0.959	[350]
Arora et al	CD	6/13	3/15	0.147	3.429	[346]
Feagan et al	CD	26/40	14/36	0.024	2.918	[347]

(Modified from Schroder et al. [348])

Methotrexate in Ulcerative colitis

Another group investigated MTX use in both UC (n=8) and CD (n=11) in a non-randomised open labelled fashion. All patients were steroid dependent. The majority of CD patients (10/11) had failed AZA/6-MP treatment and 2/6 patients with UC had received prior AZA/6-MP. Treatment

was started in an escalating fashion from 2.5 mg up to 15 mg orally until completion at week 18. Steroid reduction was successful in all those with CD who completed the study (10/11) and 2 /10 were free of steroids at the end of the study. No patients with UC had a complete response but a partial response was seen in 3/6 patients with evidence of endoscopic improvement. Side effects were mild. One patient developed headache leading to withdrawal from the study. 5 patients had mild stomatitis, 1 had mild alopecia, 1 had nausea/vomiting and 1 had cutaneous flushing. Folate supplementation was not used [349].

A trial on 72 steroid dependent IBD patients (UC n=34 and CD n=38) compared the efficacy of 1.5 mg/KG/day of 6 -MP or 15 mg/week of MTX or 3 g/day of 5- ASA. No placebo group was included. Folic acid was not used. In UC 6-MP (79% response) was superior to MTX (58% response) and 5- ASA (25 % response) in remission induction [343].

An Israeli group have performed a double-blind, randomised, and controlled trial of MTX in UC. 30 patients with confirmed UC and moderate or severe disease were randomised to receive placebo or MTX at a dose of 12.5 mg taken orally per week. Folic acid was not prescribed. Patients were treated for nine months without any dose adjustment. No significant differences were noted between the two groups in steroid use, symptom scores or side effects. Significantly more patients in the placebo group withdrew from the study [350]. The dose of MTX used is thought to be sub therapeutic. The limitations in these studies have led to MTX not finding a place in current recommendations for therapy in UC.

1.3.2.6 Mucosal Healing with methotrexate

In a letter to the editor, Manosa et al. have described their experience of serial colonoscopy in patients with CD treated with IM/SC MTX and initial dose of 25 mg per week 16 weeks followed by a dose reduction to 15 mg per week for long-term maintenance. All patients had previously received MTX, and either been intolerant (4) or unresponsive (4). All patients had been steroid dependent and all had achieved a steroid free remission. In this group, mucosal healing was seen in 5/8 patients (3 had complete healing and 2 had partial healing). All those with complete

mucosal healing had been individuals with isolated colonic disease [239]. Other data in abstract form is also referenced in their letter and may indicate a role in mucosal healing. This remains unproven at the current time.

1.3.2.7 Methotrexate toxicity

Significant toxicity leading to drug discontinuation can be limiting factor in RA therapy occurring in up to 30% of patients [351]. In IBD, side-effects appeared to be less common, leading to treatment withdrawal in 10 to 18 % [352].

Table 1-5 Toxicity of long-term Methotrexate therapy in IBD

Duration of study	Number of Patients	Leucopenia	Pneumonitis	LFT Abnormalities	Reference
69 weeks	67	0	1	0	[344]
12 weeks	19	0	0	0	[349]
16 weeks	94	0	0	7	[345]
40 weeks	40	0	0	0	[347]
9 months	26	0	0	0	[466]
9 months	30	1	0	0	[350]
6 months	20	0	0	3	[467]
16 weeks	32	0	0	1	[468]
16 weeks	9	0	0	3	[346]
17 months	70	3	0	2 (1 steatosis, 1 normal)	[278]
18 months	49		1	10 (5 steatosis,1 fibrosis,6 minor changes)	[469]

Reproduced from Fraser et al. [352]

Folate depletion mediated gastrointestinal toxicity such as nausea, vomiting, diarrhoea; abdominal pain and stomatitis are often the earliest features occurring in up to 60% [353, 354] and are manifestations of the antiproliferative effect of MTX.

Folate supplementation relieves some of these side effects without an associated fall in therapeutic efficacy. Usually, nausea may be minimised by a change in time of administration or

the route of administration (from oral to parenteral). Pancytopenia is relatively rare effecting 1 to 2% of patients and may be ameliorated by folate supplementation.

A hepatitic LFT picture is not unusual, particularly in the early stages of treatment. These are usually self-limiting and require no intervention. Some abnormalities remain persistent and may require dose adjustment or drug withdrawal. Liver fibrosis has been observed in patients with psoriasis, when treated with methotrexate and 50% of baseline liver biopsies in patients with psoriasis are abnormal. This has been attributed to excessive alcohol intake, NSAID drug use, diabetes and obesity.

Unlike psoriasis, where a cumulative MTX dosage greater than 4g contributes to irreversible hepatotoxicity [355], MTX is relatively less hepatotoxic in RA. MTX therapy in RA continued over a period of five years appears to increase the incidence of cirrhosis and liver failure with an incidence of 1: 1000. [356]. Hepatoxicity appears even rarer in IBD. A retrospective analysis of liver biopsies in patients with IBD who received a cumulative does in excess of 1.5g MTX showed cirrhotic changes in 1 out of 20 patients. LFT surveillance did not prospectively identify this patient. Currently, surveillance liver biopsies are not recommended [286] by some but are by others [212, 222] in IBD patients. The protocol recommended is the same as in RA (liver biopsy if a majority of AST values over 1 year are elevated or hypoalbuminemia is present). Pulmonary pneumonitis has been reported in IBD with an estimated prevalence of two to three cases per 100 case years. Recovery appears to be usual although mortality rates of of up to 17% have been reported [357-360].

Low dose MTX does not appear to increase the risk of malignancy and infections possibly as the drug is not incorporated into nucleic acids. [361-363]. Positive effects on homocystine metabolism [364] have been implicated in the reduced lower mortality hazard ratio (0.4) for RA patients treated with methotrexate.

Furthermore, caution in the use of MTX is recommended in individuals at risk of toxicity. These are diabetes, obesity, excessive alcohol intake, elevated baseline transaminase levels, clinically important lung disease, systemic infection, previous cancer and pregnancy [212].

1.3.2.8 Folate supplementation

Oral folate supplementation seems to diminish intracellular polyglutamation of MTX in psoriasis [365] and RA [337]. The use of supplemental folates, including folic acid and folinic acid improves adherence to therapy by reducing LFT abnormalities and GI intolerance. Folate supplementation does not appear to reduce the effectiveness of MTX in the therapy of RA. It may also have a role in the reduction of cardiovascular risk that is seen in RA patients on therapy with MTX [366].

Table 1-6 The suggested use for folates in patients taking MTX

Formulation	Indication	Dose	Benefit
Folic acid	All patients on MTX	5mg – 10 mg once a week by mouth the morning after MTX	Reduction of side effects (LFT, stomatitis)
Folinic Acid	MTX overdose or acute haematological toxicity	15 mg by mouth every 6 hours for 2-8 doses	Reversal of haematological toxicity

Reproduced from Whittle et al. [366]

1.3.2.9 The application of Pharmacogenetics to Methotrexate use in inflammatory diseases

1.3.2.9.1 MTHFR

MTHFR is the most studied in this aspect. The gene is mapped to chromosome 1p36.3 and 15 polymorphisms within the MTHFR gene have been described [307]. Of these, two non-synonymous SNPs, 677C>T and 1298A>C are the most studied. The resulting MTHFR

thermolability due to the 677C>T allows for plasma homocysteinemia and low serum folate levels [370, 371].

The MTHFR gene is mapped to chromosome 1p36.3 and 15 polymorphisms within the MTHFR gene have been described [307]. It consists of eleven exons. The 677 C>T and the 1298 A>C variants have been associated with decreased enzymatic activity. Other polymorphisms have been described and are associated with undetectable levels of enzymatic activity.

The resulting MTHFR thermolability due to the 677C>T allows for plasma homocysteinemia and low serum folate levels [370, 371]. At the 677 position 15% of Caucasians and Asians are homozygous for TT and 50% are heterozygous. With an allele frequency of 35 % in North America, this SNP causes an Alanine to valine substitution at codon 222 of the MTHFR gene [371]. At the 1298 position the mutant homozygote CC occurs in 12% of Caucasians and has an allele frequency of 33% in the same populations [372]. These mutations have important effects. The mutant homozygous *MTHFR677TT* carrier exhibits 30% of the activity of MTHFR expected of a wild type. The heterozygote genotype has 60% of wild type activity [370, 371]. The 1298A>C SNP causes a glutamine to alanine substitution at codon 429 [373]. The resulting impaired activity is not due to thermolability, is relatively mild and does not influence plasma homocysteine levels in isolation. The *MTHFR1298CC* (homozygous mutant) exhibits 60% of the enzymatic activity of a wild type genotype and 60% as a heterozygote [373, 374]. High folate states as seen in folate supplementation influences the stability of the mutant thermolabile enzyme making it more stable [375]. In folate deficient states, the homozygous mutant *MTHFR677TT* carriers show deficient DNA methylation. Similar findings have been reported *in vitro* lymphocytes from *MTHFR1298AA* wild type which has been attributed to strong LD with *MTHFR677TT* in that study population [376, 377].

The two polymorphisms are in strong LD and the genotype polymorphic at both loci is very rare (*MTHFR677TT/1298CC*) [378]. The compound hetrozygote of the two polymorphisms impart a greater reduction in the enzymatic activity than either of the two homozygous mutants on their own [373] and is relatively common in Caucasians with a prevalence of 15-23% [379]. The estimated haplotype frequencies, and the fractional contribution of each, were 677C/1298A, 0.37; 677C/1298C, 0.31; 677T/1298A, 0.32; and 677T/1298C, 0.0023 to 0.0034. Thus, a vast majority of 677T alleles and 1298C alleles are associated with 1298A alleles and 677C alleles, respectively [378].

1.3.2.9.2 Thymidylate synthase

Thymidylate synthase is inhibited directly by polyglutamated MTX and indirectly by folate cofactor depletion induced by MTX. The inhibition causes deoxythymidylate monophosphate (dTMP) depletion, mis-incorporation of the uracil cell base into DNA, chromosomal damage and subsequent cell death [368]. Three important polymorphisms have been described. A tandem repeat sequence of 28 base pairs within the 5′ untranslated region (UTR) can contain a variable number of repeats; either 2 or 3. An increasing number of sequences are associated with increasing enzyme efficiency *in vitro*. Homozygosity for the triple repeat allele *TSER *3R/*3R* is associated with greater mRNA than the double repeat allele *TSER*2R/*2R* [380]. The *3R occurs in a frequency of 50% in Caucasians and 60-80% in Asians [381]. The enhanced transcriptional activity is attributed to an additional upstream stimulatory factor (USF) protein binding site in the 3R variant. A G>C polymorphism at position 12 within the second 28bp repeat of the 3R allele gives it a transcriptional activity similar to that of the 2R allele due to interference with the function of this additional binding site [382].

A six bp deletion (TTAAAG) at position 1494 in the 3′ UTR of this gene is associated with decreased mRNA stability and lower mRNA levels are noted in homozygous carriers of the mutant deletion [383]. The polymorphism is found in frequencies of 26 % in Hispanics, 41% in Caucasians, 52% in African-Americans and 70% in Chinese [383].

1.3.2.9.3 SLC19A1

The SLC19A1 or Reduced folate carrier 1 (RFC-1) gene has been mapped to the long arm of chromosome 21 [384, 385]. The role of the reduced folate carrier in cellular transport has already been described. SLC19A1 is also responsible for the intestinal absorption of MTX and demonstrates saturation at doses of more than 15mg per day [385]. The mutant *SLC19A180GG* occurs in a frequency of 18-25% among Caucasians and Asians and is relatively rarer in Africans at 8-15%.

A SNP in the SLC19A1 gene substitutes arginine with histidine at position 80 in Codon 27. This appears to in lead to loss of SLC19A1 gene expression [386, 387].

1.3.2.9.4 AICARTase/ATIC

In animal experiments the anti-inflammatory effects of MTX appear to be mediated by adenosine receptors A_{2A} and A_3 [318]. Adenosine is difficult to investigate and estimate due to a short half-life but elegant experiments have demonstrated that adenosine receptor antagonists and adenosine inactivators such as ADA reversed the effects of MTX on leucocyte accumulation in an animal model [314, 315]. Adenosine inhibitors such as theophylline and caffeine reverse the development of adjuvant arthritis in an animal model [316] and caffeine consumption reduces the efficacy of methotrexate in humans with RA [317].

Adenosine binds specific receptors A1, A2 a, A2 b, and A3 and knockout mice studies have implied a significant role for these receptors [318]. The A1 receptor leads to immune stimulation of neutrophils, whereas the A2 a receptor leads to immune suppression [388] and is possibly the chief modulator of the anti inflammatory effects.

1.3.2.9.5 Aldehyde oxidase (AOX)

The effect of hepatic AOX on MTX forms 7 -OH MTX. This is the dominant anti folate 42 hours after administration. Like MTX, and with a similar affinity, SLC19A1 allows the cellular influx of 7-OH MTX [290]. Inside tumour cells, 7-OH MTX is a good FGPS substrate allowing exuberant polyglutamation [291, 292]. However, the resulting 7-OH derivatives are inferior DHFR inhibitors diminishing the cytotoxic efficacy of MTX [293]. The mutant AOX 3404AG has been correlated with poor response to AZA therapy [267] and appears to be a functional polymorphism.

Table 1-7 Folate Pathway Pharmacogenetics in inflammatory diseases

MTHFR	Generation of 5-methyl THF	677C>T	Thermolabile MTHFR and decreased plasma activity	1.MTX Toxicity	[470] [414] [471, 472]
				2.MTX efficacy	[410]
				3.No assoc. with toxicity/efficacy	[472, 473]
		1298A>C	Decreased activity of MTHFR	1. MTX efficacy and toxicity	[410]
				2.MTX efficacy	[414] [413]
				3.MTX toxicity	[392, 474]
				4. No assoc. with toxicity/efficacy	[473]
DHFR	DHF to THF	473G>A, 35289G>A	DNA alignment	Not assocoiated with MTX toxicity or efficacy	[410]
SHMT	Synthesis of 5,10-methylene THF	C1420T	Affects enzyme activity	MTX Toxicity	[389]
MTHFD1	10-formyl THF from 5,10 methylene THF	A1958G	Decreases Enzyme activity	MTX efficacy	[391]
TYMS	converts dUMP to dTMP	5'UTR 28-bp repeat	Increase TYMS activity	1.MTX toxicity	[389, 473]
				2. MTX poor efficacy	[423, 473]
				3. No effect on toxicity or efficacy	[412]
TYMS		6bp deletion polymorphism	Decrease TYMS stability and expression	1.MTX Toxicity	[473]
				2. No effect on toxicity or efficacy	[412]
				3. Increased efficacy	[473]

Table 1-8 Methotrexate Transporter Pharmacogenetics

Gene	Role of Gene Product	Polymorphism	Effects on Gene product	Clinical Effects	Ref
SLC19A1/ RFC-1	MTX influx	80G>A	May affect transcriptional activity of gene and reduce MTX entry	Not associated with MTX efficacy or toxicity	[390, 475]
					[423]
ABCB1	MTX efflux	3435C>T	May affect P-gp function	1.MTX efficacy	[475]
				2.Not associated with MTX toxicity	[471]
					[476]
ABCC2	MTX efflux	1249G>A	May affect enzyme activity and MTX efflux	IVS 23+56 T>C associated with toxicity	[471]
		1058G>A			
		IVS 23+56 T>C			

Table 1-9 Adenosine pathway Pharmacogenetics

Gene	Role of Gene	Polymorphism	Effects on Gene Product	Clinical effects	Ref
ATIC	Conversion of AICAR to 10-formyl AICAR	347C>G	Decreased enzyme activity	1. MTX toxicity	[389, 392]
			increases adenosine release	2. Efficacy and toxicity	[390]
				3.MTX efficacy	[391]
AMPD1	Converts AMP to IMP	C34T	Enzyme activity	MTX efficacy	[390, 391]
ITPA	Converts ITP to IMP	C94A	Enzyme activity	MTX efficacy	[390, 391]

Tables 1-7, 1-8 and 1-9 Modified from Ranganathan et al

1.3.2.9.6 Pharmacogenetic and Toxicity Indices

It is clear that a single SNP in the MTX metabolic pathway is not going to have sufficient penetrance to find application as a predictor of adverse side effects to drug therapy. Instead there is now evidence that a variety of genetic mutations can be combined to create an index with sufficient predictive power to be clinically significant.

MTHFR, TYMS, ATIC & SHMT

In a study of 214 patients with RA, 32 patients had side effects. The homozygote mutants *MTHFR 677TT, TSER*2R/*2R, ATIC 347G*G, and *SHMT 1420CC* were all significant associated with a variety of side effects. When combined together as an index, each homozygote genotype increased the chance of a side effect by 1.9 in an additive fashion [389].

AMPD1, ATIC, ITPA, MTR, MTRR & MTHFD1

Polymorphisms of adenosine pathway enzymes were studied in 205 patients with RA. The *ATIC 347CC, ITPA 94CC* and the AMPD134T allele when combined was favourable genotypes and increased the chances of a clinical response to MTX (OR 27.8). The *ATIC 347C>G* was an independent marker for toxicity [390]. In another study, 205 patients with RA were genotyped for 17 polymorphisms in 13 genes and the results were correlated with clinical outcome. The results were then validated in a further 38 patients. *MTHFD1 1958AA, AMPD1 34CC*, ITPA 94A allele and ATIC 347G allele. A scoring system was based on this model was effective in predicting 60% of responders [391].

GGH, ATIC, MTHFR, MTR, MTRR

A prospective study of 48 patients and genetic polymorphisms in SLC19A1, GGH, ATIC, MTHFR, MTRR, MTR, TSER and SHMT and MTX RBC Polyglutamate levels were correlated with clinical response and toxicity. RBC Polyglutamate levels correlated well with drug efficacy and a toxigenic index comprised of *GGH 401CC, ATIC 347GG*, MTHFR 1298C allele, *MTRR66GG* and *MTR 2756AA* was predictive of toxicity [392].

1.4 Summary and Aims:

The inflammatory bowel diseases impart a considerable health care burden and most affected individuals are in need of regular therapy. A significant number will need steroids with all their attendant risks and side effects. The use of immunomodulatory therapy namely AZA/6-MP and MTX clearly reduce steroid requirements but are themselves somewhat limited by their side effect profiles and therapeutic ineffectiveness in a significant minority. Furthermore, there is a considerable "lag time" to clinical benefits.

Alternatives are available and the newer therapies such as antiTNF drugs (Infliximab and Adalumimab) are very efficacious in a large number of patients. Importantly, they have a rapid onset of action. However, they too have a significant side effect profile and risk therapeutic failure or a loss of clinical effectiveness. Many individuals will not need these therapies, but will instead respond to immunomodulation, given adequate time to respond. Furthermore, there is evidence that early healing of the inflamed colonic mucosa is associated with improved outcomes and a reduced risk of bowel resections. Equally, the biological therapies are not infrequently, compromised by a loss of the early response. Hence the timing of biological therapy is clearly important.

As both the commonly used immunomodulatory drugs (AZA/6-MP and MTX) and the anti TNF drugs can achieve mucosal healing of the affected intestine, clearly it is important to identify those who are most likely to respond to a particular medication early so that time to mucosal healing may be minimized. However, currently available indicators do not allow a reliable identification of this group. Clinical markers of disease severity are primarily retrospective and it can be argued that it reflects existing problems and are not truly predictive. Serological markers, genetic markers and algorithms with a combination of these have been evaluated but are still of limited value.

Currently, immunosuppressive therapy is instituted relatively inflexibly. The pole position in IBD immunomodulatory therapy is occupied by AZA/6-MP. Here, the success of pharmacogenetics thus far has been predominantly in finding markers of drug toxicity. Reliable markers of clinical efficacy have not been easy to find. Second line therapy is conventionally MTX. Clearly, there

will be individuals more likely to respond to MTX than to AZA, as well as those who will not respond to either. There are a number of reports of pharmacogenetic markers of MTX efficacy in RA and cancer chemotherapy. However, the single study that examined some of these effects in IBD was probably some what underpowered to identify the relevant associations. Furthermore the important potential of an effect of folate supplementation on the pharmacogenetic markers was not examined. Therefore, the primary aim of this thesis is to examine the influence of recognized polymorphisms in the Folate Pathway and MTX efflux enzymes and to co-relate these with the response to therapy with MTX in individuals with IBD. The moderating influence of oral Folate supplementation will also be examined. This is the focus of chapters 3 and 4.

The traditional model of pharmacogenetic investigation has focused on genetic variation in metabolic enzymes. On the other hand, the effects of genetic polymorphism in innate and adaptative immunity and the influence these have on drug response are complex and obviously multifactorial. The HLA-G 14 bp ins/del polymorphisms may have an important influence on adaptative immunity and can also influence individual response to immunosuppressive therapy. This has not been examined in IBD and is the second main aim of this thesis. The effect of this polymorphism on response to MTX and AZA therapy in IBD, the examination of moderating and confounding influences, and a subsequent functional correlation is the focus of chapters 5 and 6.

2 Materials and Methods

2.1 Materials

DNA extraction and purification kits were from QIAGEN Ltd. (Crawley, West Sussex, and UK). Primers were from MWG-Biotech AG (Ebersberg, Germany). Taq DNA polymerase, agarose electrophoresis grade (standard), Agarose 1000 and low melting point agarose were obtained from Invitogen Ltd. (Paisley, UK). Restriction endonucleases and a 100bp DNA ladder were obtained from New England Biolabs Ltd. (Hitchin, Hertfordshire, UK). BigDye®terminator v3.1 cycle sequencing kit from Applied Biosystems, Warrington, UK, Agencourt®CleanSeq® from Beckman Coulter (UK) Ltd-Biomedical Research, High Wycombe, UK). The TaqMan® SNP genotyping assays and Taqman® Universal PCR mastermix were from Applied Biosystems (Warrington, UK), Lymphoprep from Axis-Shield PoC AS, Norway. FCS, Glutamine, RPMI & LPS from Sigma-Aldrich, UK. sHLA-G kit from Exbio, Prague, Czech Republic and IL-10 ELISA from eBioscience Human IL-10 ELISA Ready-SET-Go. Flow Cytomix™ Human basic kit; and the IL-8, IL-1β, TNF-α, IL-23, IL-6, IL-18 Simplex kits were obtained form Bender MedSystems®, Vienna, Austria.

2.2 Patients

A number of patients were recruited for these experiments. Ethical approval was obtained from the ethical committee of Guy's and St Thomas' NHS Foundation trust for the MTX and AZA cohorts. Ethical approval for the additional pharmacogenetic work and cell culture experiments was granted by Bexley and Greenwich LREC (06/Q0707/84).

For the MTX pharmacogenetics experiments, patients were recruited retrospectively from three tertiary referral centres in the United Kingdom. These included Guy's and St Thomas's Hospital in London, Addenbrooke's Hospital, Cambridge and the John Radcliffe Hospital, Oxford. A significant proportion of these patients have formed part of two previously published studies concerning clinical outcome on MTX [398, 399]. Patient details were obtained from an IBD database and cases were selected on the basis of age more than 16 years. The cases were seen in

gastroenterology or surgical clinics. Clinical details were collected by case note review by four independent assessors. The author of this work reviewed all the patient data from the Addenbrooke's cohort and the Guy's and St Thomas's cohort. Details about the assessment of clinical response and side effects are available in chapter 3.

Patients studied for the pharmacogenetic influences on AZA response formed part of a larger prospective multicentre study of AZA for the treatment of IBD. It was designed to assess the impact of pre-treatment TPMT genotype and activity and subsequent red cell TGN levels on clinical outcome. The initial study reported on 215 individuals and the experiments reported here have examined the individuals who tolerated medication for a minimum period of three months and those for whom DNA was still available. (n=99). Further details on this cohort are available in Chapter 5.

Patients studied for the effect of the HLA-G polymorphism on susceptibility to IBD comprised of 920 cases with CD, 654 with UC and 665 controls. The cohort has previously been reported in a CD susceptibility study [436]. A multicentre research ethics committee approved the study of IBD susceptibility genotype in this cohort and the patients were recruited from Guy's and St. Thomas's Hospital, London, St. Mark's Hospital, London and the Royal Victoria Infirmary, Newcastle. Further details of this group are in Chapter 5.

For the functional experiments, seventeen Caucasian, female volunteers, aged between 25 and 55 years, were recruited. None of the volunteers had co existing medical conditions or were on any medications. After informed consent, phenotype details and whole blood was obtained. Details of this group are in Chapter 6.

Population controls were obtained from the 1958 British Birth Cohort, which includes all subjects born between 3 and 9 March 1958 in England, Scotland, and Wales (National Child Development Study: (http://www.cls.ioe.ac.uk).

2.3 Molecular Biology

DNA sequences were obtained from GenBank. With the help of Copy-DNA (cDNA) the genomic sequence was examined for introns, exons and sequence variants. DAPSA software (DNA and Protein sequence Alignment Software, Dr E. Harley, University of Cape Town, South Africa) was used to search and edit the sequences before identifying potential restriction endonuclease sites for digestion.

2.3.1 DNA Extraction from whole Blood

DNA was extracted from whole blood using QIamp® DNA Mini Kit 250 (QIAGEN Ltd., Crawley, West Sussex, UK). From 200µl of whole blood, it provides 6µg of DNA of around 20-30kb in length. Blood samples were taken from consenting patients in an EDTA tube and frozen. After thawing, 200 µl of blood was lysed by 20µl QUIAGEN Protease enzyme and 200µl of Buffer AL. Following a brief vortex, protein denaturation was achieved by incubation for 10 minutes at 56°C. 200µl of 100% v/v Ethanol was added and the sample was vortexed again. Following this, it was transferred to a spin column placed in a 2 ml collection tube. Centrifugation was performed at 6000×G for 1 minute. This allows the adsorption of the DNA onto the silica-gel QIAmp membrane which is not released during subsequent washing steps. The DNA is then eluted by centrifugation at 6000×G for 1 minute of the spin column with 200µl of AE Buffer into a 1.5ml collection tube. The final DNA product is mixed with 50µl of 1×TE buffer (10mM Tris.Cl, pH 8.0, 1mM EDTA, pH 8.0) to inhibit DNAases and stored at -20°C.

2.3.2 Polymerase Chain Reaction

The introduction of the concepts of the polymerase chain reaction (PCR) revolutionised molecular biology. It is an elegantly simple concept that allows the selective amplification of specific DNA sequences from the genome. This is done by targeting of the required sequence. Hence, prior knowledge of this required, which in most cases is obtained from GenBank. The required sequence is flanked by primers that are designed using some important “rules”

Rules of Primer Design:

- The fragment should be around 20- 30 base pairs long
- Tandem repeats of one or more nucleotides should be avoided especially guanine for more than 4 bases.
- Sequences prone to secondary structure formation such as hair pins must be avoided.
- The two primers should not be complimentary at the 3′ end so that primer-dimers (complexes formed by the mutual binding of the two primers) may be avoided.
- The Ideal G+C content should be around 40% and never more than 70%.

Accidental matching by an unintended strand of DNA to the two primers chosen in keeping with the above guidance is unlikely and this technique is therefore extremely specific.

When this reaction is provided with a heat stable DNA polymerase enzyme and precursors of DNA (dATP, dCTP, dGTP, dTTP), the target sequence that is flanked by the two primers is reproduced in reverse (complementary strand). These serve as templates for further DNA synthesis and after around 25 cycles of replication around 10^5 copies of the targeted sequence will be available.

This reaction is usually divided into a number of steps. At the initialisation step, heat is applied to a temperature of 94 to 96°C and activates the Taq enzyme. This is followed by the denaturation Step where a temperature of 94 to 98°C is applied for 20 to 30 seconds to denature the hydrogen bonds and provides providing single DNA strands. These will provide the templates for replication. Then, in the annealing Step, the reaction is cooled to 50 to 65°C for 20 to 40 seconds. The primers pair with the single-stranded DNA template. If the primers are well selected, stable hydrogen bonds are formed between the primers and the template sequence. In the next step, the extension/elongation occurs and the DNA polymerase creates a new complimentary DNA strand, in a 5′ to a 3′ direction. The final elongation step occurs at a temperature of 70 to 74°C and lasts for five to 15 minutes after the last PCR cycle to ensure complete extension of single-stranded DNA (Adapted from Strachan and Read, Human Mol Gen 2) [478] (Figure 2-1).

A PCR blank and genotype controls were included in each series of PCR reactions.

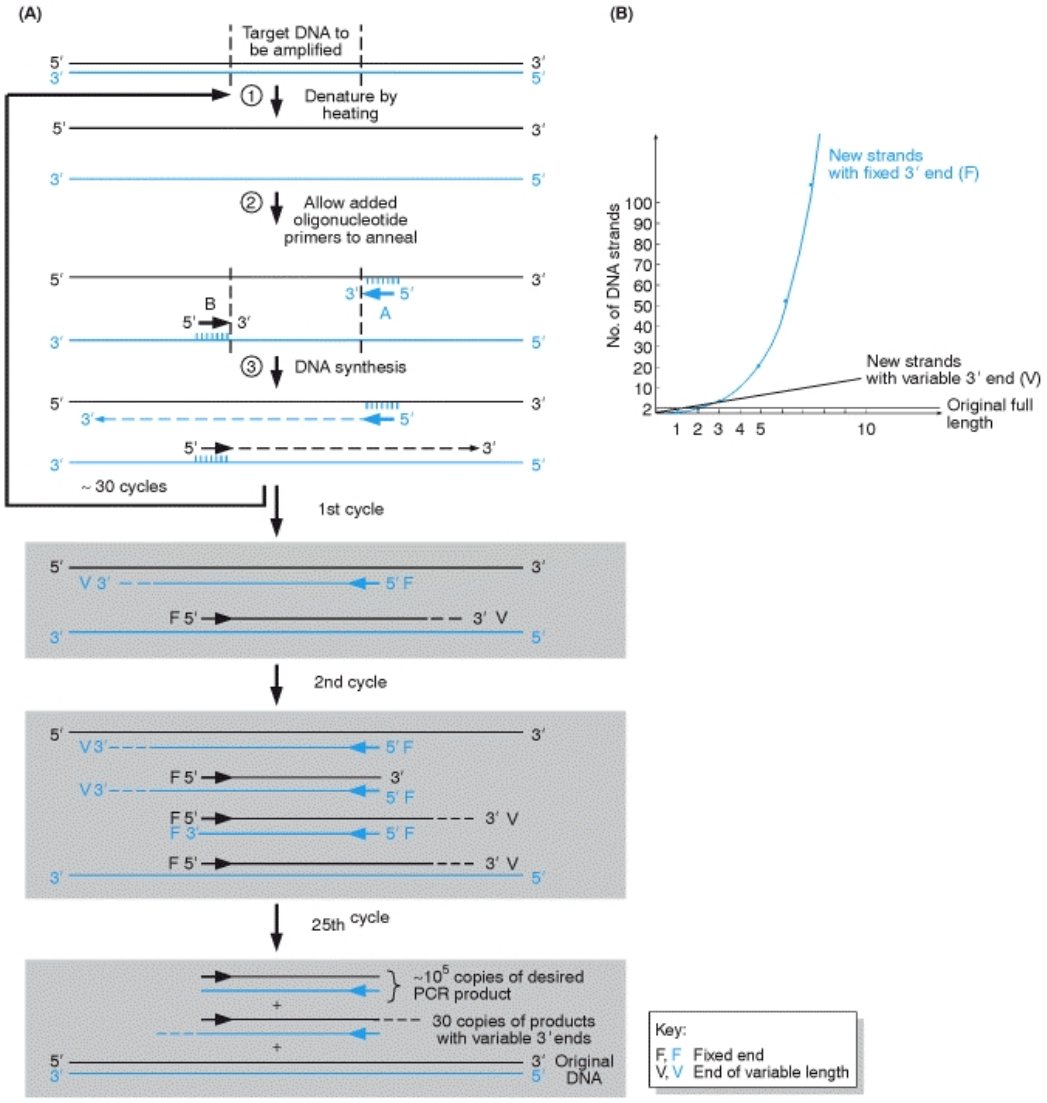


Figure 2-1 The technique of PCR (reproduced from Strachan and Read, Human Molecular Genetics 2)
[478]

This project has used four PCR based methods to identify known Mutations.

- Mutation Detection with TaqMan™ allelic discrimination assay
- Restriction Fragment Length Polymorphisms (RFLP)
- Allelic Discrimination, Allele specific PCR
- Sequencing using fluorescent labelling and automated detection

2.3.3 The TaqMan® Assay

Taqman assay is a process that allows for automated large volume genotyping. Standard PCR is used to amplify the product but for allelic discrimination, a Taqman probe is used. This is composed of two types of fluorophoric proteins. These are the reporter at the 5′ end and the quencher at the 3′ end. They are located close to each other on the probe and in their position of proximity; fluorescence resonance energy transfer (FRET) inhibits the activity of the reporter. Because the 3′ end is blocked, the probe cannot prime any new DNA synthesis. When the polymerisation chain reaction takes place, the sequential addition of nucleotides over the DNA template separates the quencher form the reporter and light is emitted. This is detected and analysed by the 7900HT Fast Real-Time PCR system (Applied Biosystems) (Figure 2-2).

The assay reagents consist of a 40× or a 80× mix of un labelled PCR primers and Taqman® probes (FAM™ and VIC® dye labelled). They require the use of a mastermix that comprises of Taq polymerase, nucleotides and the required chemical conditions. We have used the Taqman® Universal PCR mastermix.

Procedure

A 5µl reaction is formed of Taqman® Universal PCR mastermix 2.5 µl, 40 × Assay mix 0.125 µl, Genomic DNA diluted in dH_2O to 2.375µl. Based on previous RFLP, control samples of all 3 genotypes were included on a 96 well plate. The annealing is at 50° C for 2 minutes followed by extension/elongation at 92° C for 15 sec, 60 C for 1 minute for 40 cycles. The final elongation is at 95° C for 10 minutes. The product is then loaded into the 7900HT Fast Real-Time PCR system which produces a colour coded result (Figure 2-3).

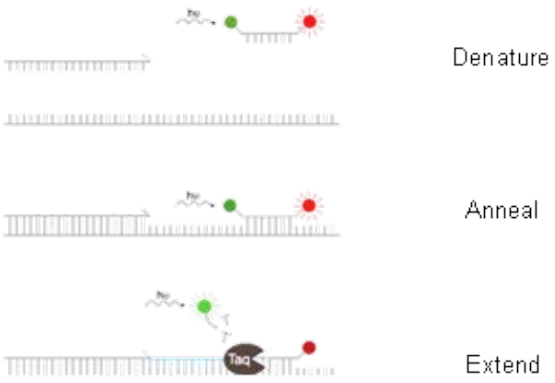
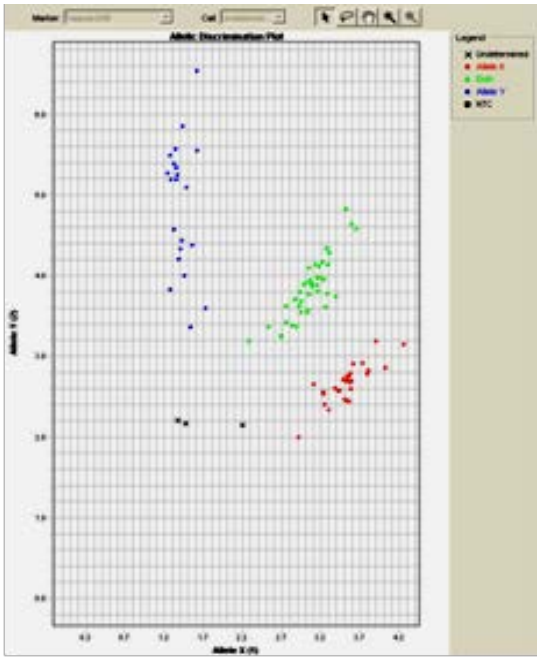


Figure 2-2 Mechanism of action of the TaqMan® SNP assay probes reproduced from (www.probes.com)



This was obtained in IL-10- 1082A>G genotyping. The AA is demonstrated as blue dots, the AG heterozygote as green dots and the GG homozygotes as red dots. The two black "x" are blanks. One sample could not be assigned a genotype. On review by sequencing, it was GG.

Figure 2-3 Allelic discrimination plot

The following probes were used in the genotyping of the studied polymorphisms.

Table 2-1 Taqman® Probes used in genotyping

SNP	RS Number	Probes
ATIC 347C>G	rs2372536	C allele 5′-FAM-TCCAGGTGTAACTGTT-MGB -3′
		G allelet 5′-VIC-CCAGGTGTAAGTGTTG-MGB -3′
MTHFR677C>T	rs1801133	C Allele : 5′-VIC-ATGAAATCGGCTCCCGC′3
		T allele : 5′-6 FAM-ATGAAATCGACTCCCGC′3
MTHFR1298A>C	rs1801131	A-allele : 5′-VIC-AGTGAAGAA AGT GTC TTT MGBNFQ-3′
		C-allele : 5′-6-FAM-AGT GAA GCA AGT GTC TT MGBN FQ-3′
SLC19A1 80G>A	rs1051266	G allele: 5′ -VIC-CACGAGGCGCCGC - 3′
		A allele: 5′ -6 FAM-CGAGGTGCCGCCAG- 3′
IL-10- 1082G>A	rs1800896	G allele : 5′VIC-TTCCCCCTCCCAAAG- - 3′
		A allele : 5′- 6FAM-TTCCCCTTCCCAAAGA- 3′
AOX1 3404A>G	rs55754655	A allele : 5′VIC-AAGCATTATCCTTTCAG- - 3′
		G allele : 5′- 6FAM-TGAAAGCATTACCCTTTCAG- 3′

The results were confirmed in a selection of 25 samples by RFLP. The techniques used for this are described below. All of the results were obtained by TaqMan assays were confirmed by RFLP in the 25 selected samples.

2.3.4 Restriction Fragment Length Polymorphism (RFLP)

RFLP is a technique wherein a restriction endonuclease specifically recognises and cleaves double stranded DNA at a sequence specific site. A sequence variant may create or destroy a restriction site. PCR is used to amplify the DNA fragment containing the altered site and digestion with the predetermined enzyme splits the product at the restriction site yielding smaller fragments of a predicted size. The resulting fragments are of varying length and discriminate on electrophoresis. This allows identification of the homozygous and heterozygous and wild type genotypes with great accuracy.

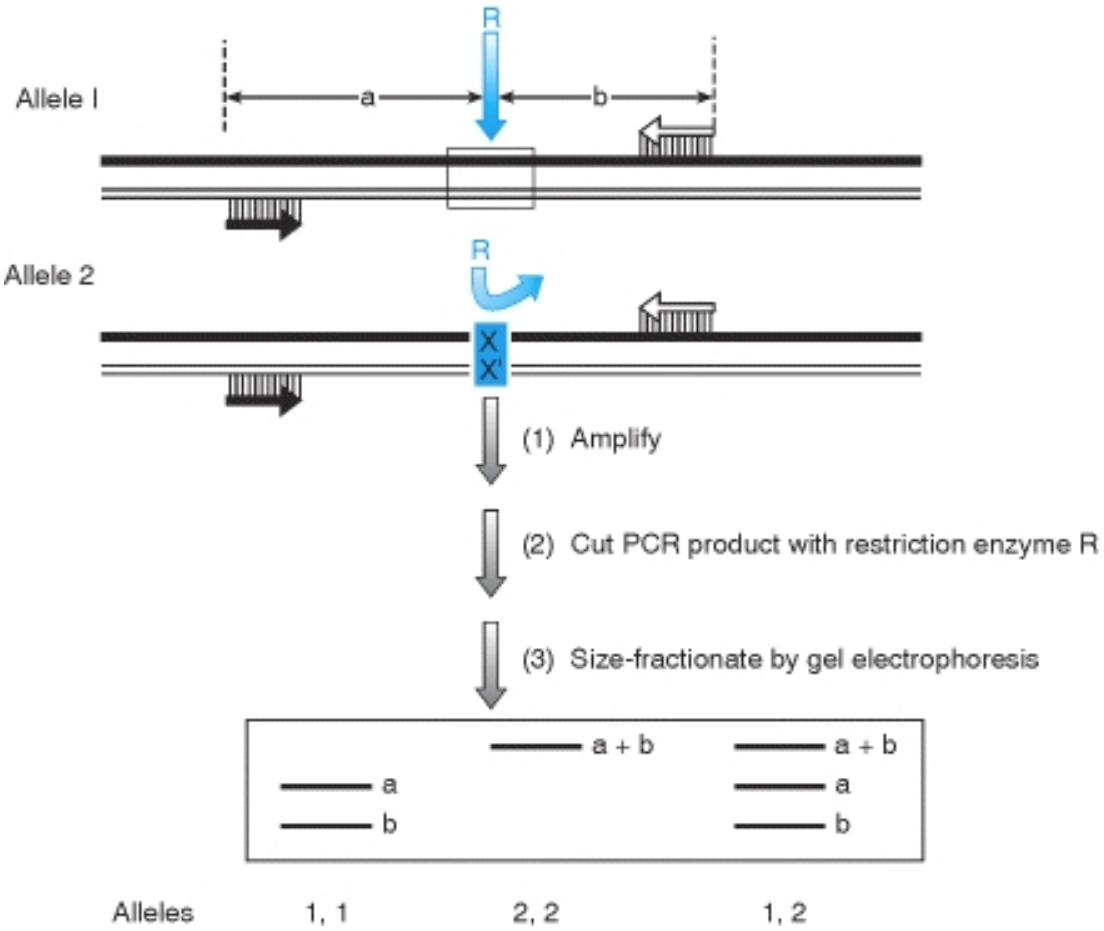
Incompatibility between the PCR buffer and the digestion enzyme buffer some times caused incomplete digests and poor discrimination on gel electrophoresis. This was resolved by a dialysis step on the PCR product before digestion. The process was performed on Millipore VSP filters (Millipore-Waters, Elstree, UK) floating in a water bath with deionised water. Passive dialysis was performed for 3 minutes followed by retrieval and subsequent digestion.

Lambda (λ) DNA is an *E.coli* bacteriophage which has restriction sites that are recognized by a variety of restriction enzymes. It was used as digest control for the experiments. Bovine serum albumin (BSA) was used in some digests according to the enzyme manufacturers' recommendation to prevent enzyme adhesion to reaction tubes and pipette surfaces. The processes described above are labour intensive and have been largely replaced by rapid throughput real-time methods. They were used in this project for confirmation of the results of TaqMan™ SNP genotyping assays.

Restriction enzymes were used in the below recipe to create a master mix with 10× supplied buffer 1.6µl, PCR product 14.4 µl and 5 U of the supplied Restriction Enzyme. A master mix was made in order to avoid working with small volumes. From this 2µl containing the required 5U was used. Samples were then incubated for the period suggested by the suppliers and then separated on an agarose gel.

Gel Electrophoresis

The PCR products were separated on 2% (w/v) standard agarose gel run in 1×TAE electrophoresis buffer containing 1µl ethidium bromide (EtBr at 1mg/ml). EtBr intercalates between the complementary strands of a DNA molecule and fluoresces under UV light. The gel is poured into a casting tray with a comb and allowed to set for 20 minutes. The PCR product (10 µl) is mixed with 4µl of a loading buffer and loaded into the well. A 100bp DNA ladder was used to estimate fragment sizes. Gels were run at a constant voltage and the results were examined under UV light. Appropriate photographic evidence was obtained.



(Reproduced from Strachan and Read, Human Molecular Genetics 2) [478]

Figure 2-4 The technique of RFLP

Table 2-2 Primers, PCR conditions and restriction enzymes used for the amplification and detection of MTHFR, SLC19A1 and ATIC polymorphisms. Separation at 90V for 35 minutes for MTHFR and 100V for 60 minutes for the others

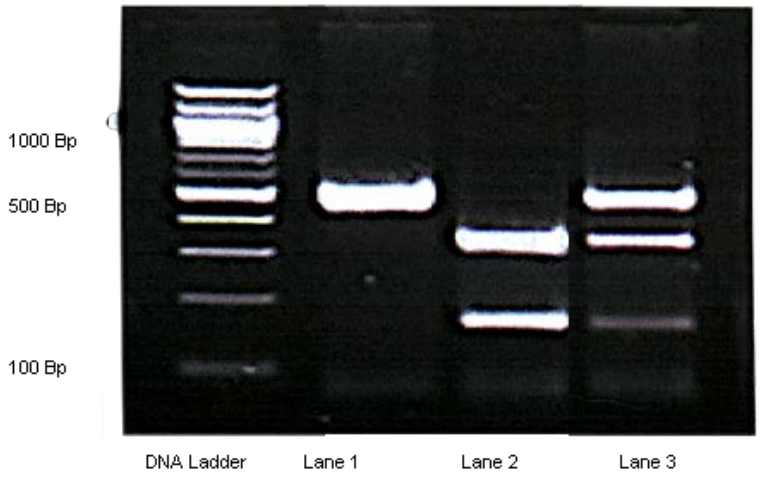
Allele	Forward Primer	Reverse Primer	Thermocycler profile	Restriction enzyme	Agarose gel
MTHFR 677 C>T	5′- CCCAGCCACTCA CTGTTTTAGTTC- 3′	5′- CCAAAGTACAACAAA CCCCTCAAC - 3′	94°C 2 min, 94°C 30 s, 48°C 30 s , 72°C 30 s: 30 cycles and then 72°C for 10 min	*Hinf*I	10 µL PCR product on 2% standard agarose
MTHFR 1298A>C	5′-CATGTGGTGGCACT GCCCTCTG - 3′	5’-CGAGAGGTAAAGAACA AAGACTTCAGCGACAC - 3′	94°C for 2 min, 94°C for 30 s, 50°C for 30 s, 72°C for 30 s: 35 cycles, then 72°C for 10 min.	*Mwo* I	10 µL PCR product on 3% agarose 1000
SLC19A1 80G>A	5′- CCTGACTC CACCCCTCCTTCC-3′	5′-TGCTCCCGCGT GAAGTTCTTGTC -3′	94°C for 2 min, 95°C for 20 s, 64°C for 20 s, 72°C for 30 s: 35 cycles. 72°C for 10 min	*Dra* III.	10 µL PCR product on 3% agarose 1000
IC 347C>G	5′-CCTTTGTAAAGACAGTGG CTTCTCCAGCAGTAA -3′	5′-AATTATAGTAATCCCAAAAC ACAATCCAGAAGTAG -3′	94°C for 1min and 94°C for 30s, 54°C for 30s, 72°C for 30 sec: 35 cycles and 72°C for 10min.	*AlwN*I	10 µL PCR product on 2.5% agarose 1000

2.3.4.1 MTHFR 677 C>T

The 677 C>T mutation creates a *Hinf*I site. The restriction site is 5'…G^ANTC…3' and 3'…CTNA^G…5'. The 579 bp fragment includes a second non-polymorphic *Hinf*I site creating an extra 72 bp control in all samples when digestion is complete at 37°C overnight. The fragment sizes are shown in (Table 2-3) and the photographic gel demonstrates the findings (Figure 2-5).

Table 2-3 Allelic typing after size fractionation and gel discrimination (MTHFR 677 C>T)

DNA fragment size	MTHFR 677 CC	MTHFR 677 CT	MTHFR 677 TT
507 bp	+	+	_
342bp	_	+	+
165bp	_	+	+
72bp	+	+	+



HinfI Digestion has produced the above results. Lane 1: Wild type (677 CC), Lane 2: Homozygous (677TT), Lane 3: Heterozygous (677CT)

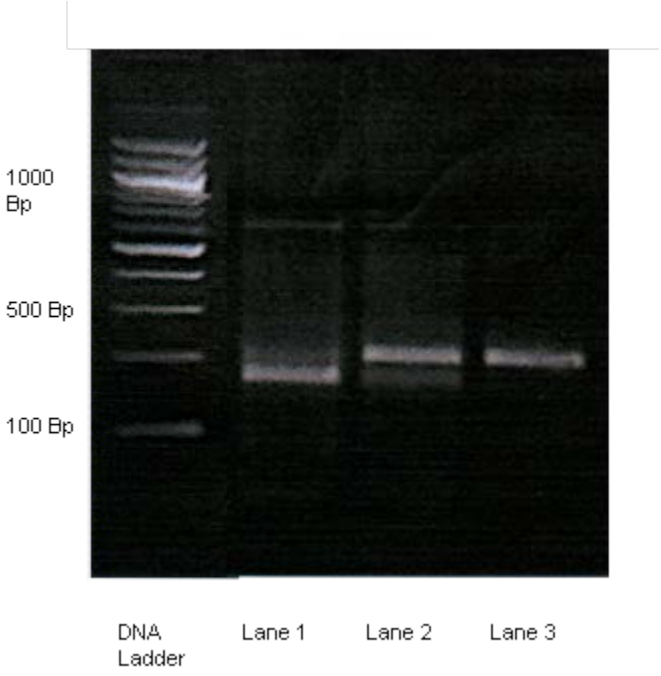
Figure 2-5 Gel image of RFLP of the MTHFR 677 C>T mutation

2.3.4.2 MTHFR 1298A.>C

This was more complex. It is uncertain if the silent polymorphism at 1307C>T is recognized by the well described restriction enzyme *Mbo*II at 37°C overnight that was used to identify the 1298A>C mutation. This would create false positives. Hence a mismatch reverse primer was designed with the purpose of using *Mwo*I. A mismatch reverse primer was used that had a complementary sequence but differed at AA→GC. This allows digestion by *Mwo* I when the 1298 A>C mutation is present. The recognition site is: 5'…GCNNNNN*NNGC…3' and 3'…CGNN*NNNNNCG…5'.

Table 2-4 Fragment sizes after gel electrophoresis- MTHFR1298A>C

DNA fragment size	MTHFR 1298AA	MTHFR 1298CC	MTHFR 1298AC
211 bp	+	-	+
177 bp	-	+	+
34 bp	-	+	+



Lane 1 : Homozygous (1298CC), Lane 2: Heterozygous (1298AC), Lane 3: Wild type (1298AA)

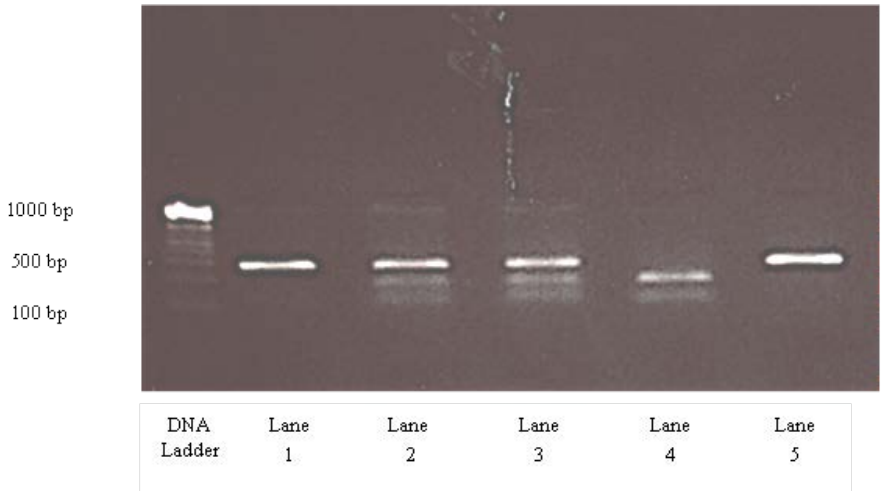
Figure 2-6 Gel image of RFLP of the MTHFR 1298 A>C mutation

2.3.4.3 SLC19A1 80G>A

Overnight Enzymatic digestion was performed with *Dra* III at 37ºC for 2 hours. It is created from an *E.coli* strain that carries the DraIII gene from *Deinococcus radiophilus*. The recognition site is 5'…CAGNNN*GTG…3' and 3'…GTG*NNNCAC…5'.

Table 2-5 Fragment sizes seen after *Dra*III digestion in RFLP for the SLC19A1 80A>G mutation

DNA fragment size	RFC-1 AA	RFC-1 AG	RFC-1 GG
150 bp	+	+	-
109 bp	-	+	+
67 bp	-	+	+



Three fragments are produced 150, 109 and 67bp.

Lane 1 and 5: Homozygous (GG), Lane 2 and 3: Heterozygous (AG), Lane 4: Wild type (AA) and 100 bp ladder

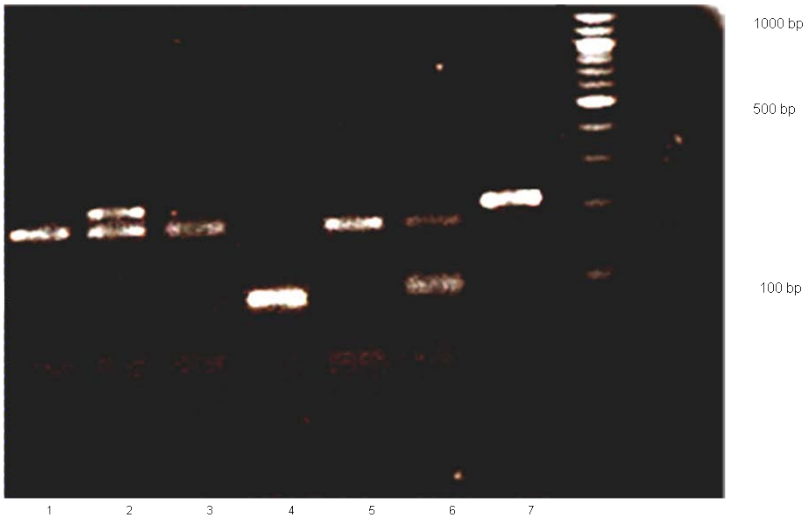
Figure 2-7 Gel electrophoresis of the SLC19A1 80A>G polymorphism

2.3.4.4 ATIC 347C>G

Microdialysis was performed of the product and then digestion was performed with *AlwN*I at 37°C overnight. The digestion site is 5'…CAGNNN*CTG…3' and 3'…GTC*NNNGAC…5'.

Table 2-6 Fragment sizes seen after *AlwN*I digestion in RFLP for the ATIC 347C>G mutation

DNA fragment size	ATIC CC	ATIC CG	ATIC GG
207 bp	+	+	-
172 bp	-	+	+
35 bp	-	+	+



Three fragments are produced 207, 172 and 35bp. The 35 bp fragments are seen very faintly. Lane 1, Lane 3 and Lane 5: GG, Lane 2: CG, Lane 7: CC, Lane 4: control DNA.

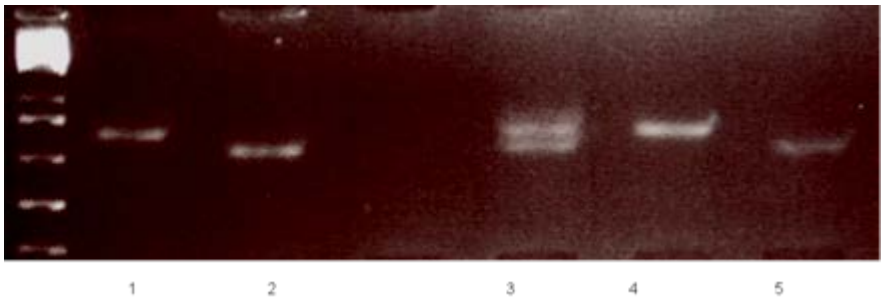
Figure 2-8 Gel electrophoresis image of the ATIC 347C>G polymorphism

2.3.5 Allelic Discrimination by sizing assay

Small sequential insertions or deletions of nucleotides can be very simply detected by targeting primers to flanking regions of the polymorphism and then distinguishing the normal and mutant alleles by size on polyacrylamide or agarose gels.

2.3.5.1 HLAG 14 bp insertion /deletion polymorphism

The Forward Primer was RHG4 5′-GGAAGGAATGCAGTTCAGCATGA -3′ and the reverse Primer was GE14HLAG 5′-GTGATGGGCTGTTT AAAGTGTCACC -3′.
Thermocycling conditions were 92°C for 5 minutes, and 30 cycles at 92°C for 30 seconds, 64°C for 1 minute, and 72°C for 2 minutes, with a final elongation step of 72°C for 10 minutes. Reactions were set up in a 25µl mixture containing 100 ng of genomic DNA, 0.2 mmol/l NTPs, 1.5 mmol/l MgCl2, 10 pmol of each primer, and 1U of Taq polymerase.The amplified products were visualized by electrophoresis on a 2.5% agarose gel (Invitrogen, Paisley, Scotland, UK) containing ethidium bromide (0.5 µg/ml) and were run at 100v for 120 minutes. Fragments of 224 bp and 210 bp were produced.



Lane 1: 14bp ins/ins, Lane 2: 14bp del/del, Lane 3 : 14 bp ins/del, Lane 4: ins/ins, Lane 5: del/del

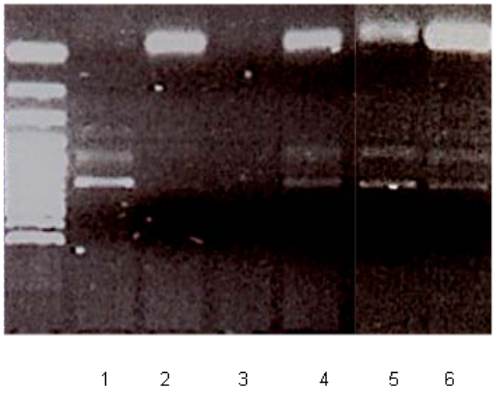
Figure 2-9 Gel image of the HLA-G 14 bp ins/del polymorphism

2.3.5.2 Thymidylate synthase 3′ un-translated region 6 base pair insertion/deletion polymorphism (TYMS 3′ (UTR) 6bp ins/del)

Samples were amplified twice. Both amplifications shared the same forward primer and differed in the reverse primer. Forward Primer was 5'-ATTACAA CAGGTCGTACAATTATGGC-3' The "positive" 5'- CTTTATTATAGCAACATATAAAACAACTATAACT-3' primer was designed to anneal when the 6-bp sequence was present. The "negative" 5'-TTTATTATAGCAACATATAAAACAACTATA AAGT-3' reverse primer annealed specifically to the DNA sequence when the 6-bp fragment was absent. Thermocycler Profile was 94° C: 1 min, 94° C: 45 s, 54° C 30 s, 72° C 30 s: 35 cycles and 72° C: 10 minutes, 1 cycle. The fragments amplified with each set of primers were approximately 673-bp long. Each sample was run on consecutive lanes on a 2% standard agarose gel and scored for the presence or absence of the 673-bp band.

Table 2-7 Fragment sizes for the TS 6bp ins/del mutation

	Insertion/Insertion	Insertion/Deletion	Deletion/Deletion
6bp Positive band	+	+	-
6bp negative band	-	+	+



Lane 1 and 2: 6bp ins/del, Lane 3 and 4: del/del, Lane 5 and 6: ins/ins

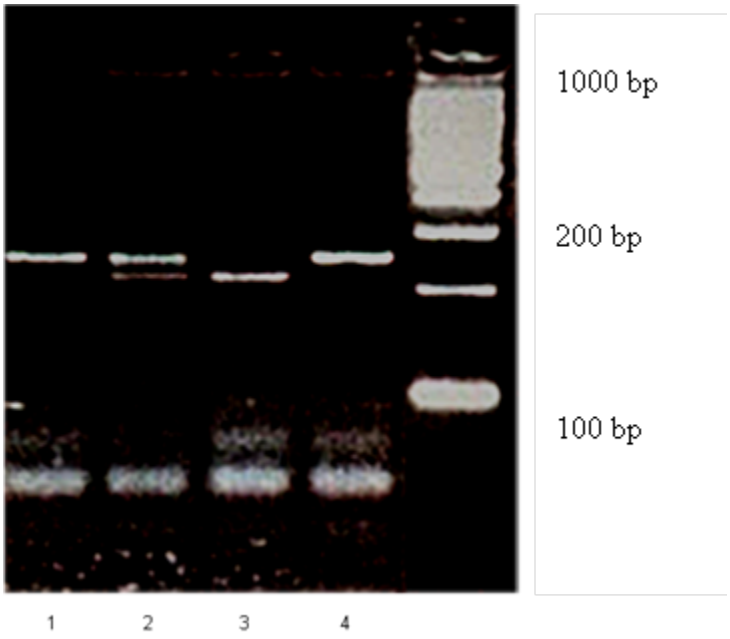
Figure 2-10 TS 6bp ins/del gel image

2.3.5.3 Thymidylate synthase enhancer region (TSER) 28 base pair tandem repeat TSER 3R/2R

PCR was performed on genomic DNA with forward primer: 5′ GTGGCTCCTGCGTTTCCCCC-3′ and reverse primer: 5′ - GCTCCGAGCCGGCCACAGGCATGGCGCGG-3′. The thermocycler profile was 94° C: 1 m, 94° C: 1 min, 60° C 30 s, 72° C 30 s: 35 cycles
72° C: 10 minutes, 1 cycle. The resulting fragments were separated on a 3% agarose gel and two products are seen.

Table 2-8 Fragment sizes for the TSER 3*R/2*R mutation

DNA fragment size	TYMS 3R/3R	TYMS 3R/2R	TYMS 2R/2R
220 bp	Absent	Present	Present
248bp	Present	Present	Absent



Lane 1 and Lane 4: 3R/3R, Lane 2: 3R/2R, Lane 3: 2R/2R

Figure 2-11 TSER 3*R/2*R gel image

2.3.6 Sequencing

The IL-10 promoter region containing the -1082 A>G (rs1800896), -819 C>T (rs1800871) and -592 C>A (rs1800872) polymorphisms was amplified using MolTaq Thermostable DNA polymerase 5U/µl (Molzym GmBH&Co. KG, Bremen, Germany), in a total volume of 50 µl PCR reaction. Primers were designed using the Primer3 v.0.4.0 website [393].

0.5 µM forward primer 5'-CACAAATCCAAGACAACACTACTAAGG-3' and 0.5 µM reverse primer 5'-ATCCTCAAAGTTCCCAAGCAGC-3' was used in a thermocycler profile of 94° C: 1 minute, 94°C for 30 s, 54°C for 30 s, and 72° for 1 min 94: 35 cycles, 72° C: 30 sec, 72° C and a finally a 10 minutes cycle. The amplified PCR products were purified using QIAquick®PCR purification Kit (QIAGEN Ltd, Crawley, West Sussex, UK). This is a simplified centrifuge assisted purification process using a bind- wash- elute procedure. It removes primers, nucleotides, enzymes, and other impurities. The binding buffer is added directly to the sample and the mixture is added to quick spin column. The high-salt conditions in the buffer causes nucleic acids to adsorb to the silica-gel membrane. After centrifugation, the DNA is eluted with the low-salt buffer provided in the kit.

Automated DNA sequencing was performed on the amplified product of the PCR reaction. Four separate fluorescent dyes are used as labels for the base specific reactions. After annealing, the Taq polymerase elongates the fragment with dNTP. If the dye terminators that are also present are incorporated, they prevent further extension and leads to strands of varying length. During electrophoresis, a laser beam is focused on the gel and this causes the dyes to fluoresce at different wavelengths for the different dyes. This gives a series of fragments of differing bp lengths that are assembled by specialized software to provide the DNA sequence (Figure 2-12).

Sequencing reactions were performed using the BigDye®terminator v3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK) and run on an ABI PRISM 3130x1 Genetic analyser (Applied Biosystems, Warrington, UK).with an additional two nested primers. These were forward : 5'-GGGTGGAAGAAGTTGAAATAACAAGGAA-3' and reverse 5'-

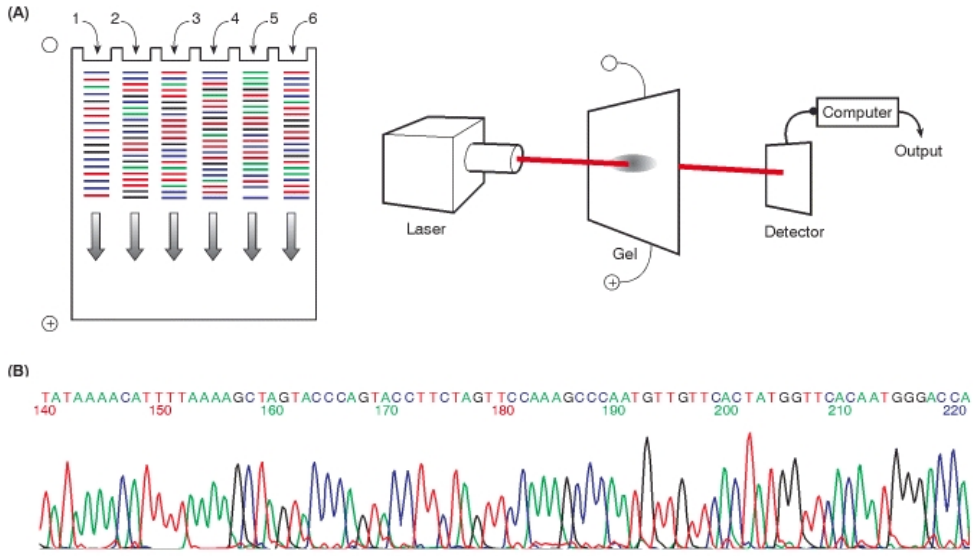
AAGTGCAGACTACTCTTACCCACTTC-3'. The kit contains the Ready Reaction Premix, and Big Dye sequencing Buffer.

The Recipe used is of Ready reaction premix 2.5× 4µl, Bid Dye Sequencing Buffer 5× 2µl , Primer 3.2 pmol, Template DNA 2, The final volume was 20µl.

Sequencing reactions were cleaned using Agencourt®CleanSeq® (Beckman Coulter (UK) Ltd-Biomedical Research, High Wycombe, UK)

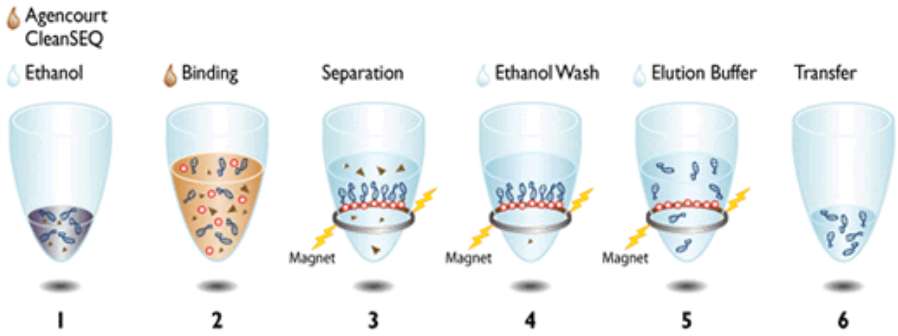
The technique was as follows, Add Agencourt CleanSEQ reagent and ethanol to sequencing reaction, bind sequencing products to magnetic beads, separate sequencing products from contaminants with magnetic field, wash with ethanol, elute from magnetic particles and then transfer away from magnetic beads (Figure 2-13).

Sequences were analysed by Mutation Surveyor Local v3.20 (Softgenetics LLC, State College, PA 16803 USA).



(Reproduced from Strachan and Read, Human Molecular Genetics 2) [478]

Figure 2-12 Automated DNA sequencing using fluorescent primers



After binding with the magnetic beads separation is performed in a magnetic field. Subsequent ethanol washing cleanses and the beads are eluted and transferred out.

Figure 2-13 :Cleansing technique for the Sequencing Reactions

Reproduced from Agencourt®CleanSeq® (Beckman Coulter (UK) Ltd-Biomedical Research, High Wycombe, UK) product insert.

2.4 Cell Culture Experiments

Peripheral Blood Mononuclear cells

PBMCs were selected as the cells of choice as the aim was to examine the effects of pharmacologically relevant doses of 6-MP and MTX on the innate immune response in cytokine expression (particularly, IL-10) and sHLA-G. Furthermore, certain cytokines may be very similarly expressed in PBMCs from patients with IBD and normal individuals. PBMCs are a mixture of monocytes and lymphocytes and the effects of stimulation and drug exposure across the whole spectrum of cells implicated in HLA-G function could therefore be examined. This is, as described in the introduction, wide ranging. Furthermore, PBMCs are readily available from healthy donors. Lamina propria intestinal mononuclear cells are a very attractive option, however supply is unpredictable particularly as the aim was to study the effects in healthy individuals who were not known to have inflammatory disease, malignancy or known to be pregnant. Hence,

PBMCs were the preferred option. The report of IL-10 expression being similar between PBMCs from IBD patients and normal individuals [394] further supports the choice.

Monocyte populations in individuals with CD differ from classical monocyte populations in that they have a mature and pro inflammatory phenotype characterized by high TNF-α and low IL-10 levels. They are characterized by cell surface markers $CD14^{HIGH,}$ $CD16^{+}$, $CD56^{+}$ [395]. Such characterization of subpopulations has not been performed in this study, but cytokine expression levels on LPS stimulation appear consistent with those seen in inflammatory conditions. There are therefore differences between LPMCs and PBMCs in patients with IBD [396] and there are differences between PBMCs in individuals with IBD and those from individuals without inflammatory disease. On LPS stimulation of normal PBMCs, many similarities between these populations emerge. *In vitro*, they are immunologically active and comprise of T lymphocytes, monocytes. Hence, the use of PBMCs from normal individuals stimulated with LPS, a bacterial wall component that is found in a number of pathological Gram negative bacteria as the populations of cells for the *in vitro* experiment appears reasonable.

Whole blood was collected from healthy Caucasian female volunteers, not taking any medication. As there is a considerable influence of soluble HLA-G in pregnancy, we specifically selected individuals who could be sure of a menstrual period within the last month. Men have lower sHLA-G levels than women and were therefore not recruited. As previously described, a number of immunologically active drugs influence sHLA-G levels and so therefore, all individuals were not taking any medications. History of coexisting medical conditions was elicited and individuals with active inflammatory conditions and diabetes were not recruited.

2.4.1 Preparation of PBMCs

The blood samples were collected into lithium-heparin tubes and within 4 hours were diluted in an equal volume of Dulbecco's Phosphate-Buffered Saline DPBS (GIBCO 14190, Invitrogen). 30 ml of blood was layered onto 15ml of FICOLL-Hypaque. Centrifugation was performed at RCF 800 G for 20 minutes at 20°C. The PBMCs at the interface between the serum and the blood was carefully aspirated. The rest was discarded. DPBS was then added to make up the volume to 45 ml. Centrifugation was repeated at RCF 1400 G for 10 minutes at 20°C. The supernatant was then carefully aspirated and the cell pellet was re-suspended in 15ml dialysed fetal calf serum 10% + RPMI +1:500 glutamine (4mM L-glutamine).

2.4.2 The Drug Dilutions

Serial dilutions of both 6-MP and MTX were required. MTX has a molecular weight of 450 and 6-MP a molecular weight of 170. The molecular weight was used to prepare drug concentrations of 1 μM, 0.5 μM, and 0.25 μM. The solutions were made in DMSO. Previous experiments have demonstrated that the 1μM concentration of 6-MP is most consistent with therapeutically significant concentration of 6-TGN levels in co-incubated PBMCs. Levels of 10 μM and greater are associated with apoptosis [254]. Hence 1 μM was the maximal dosage evaluated. FCS, glutamine, RPMI & lipopolysaccharide (LPS) were all from Sigma-Aldrich, UK. The LPS solution was made up to 50 μg/ml.

2.4.3 Cell Culture

Cell culture was performed on a 16 well plate each of which contained 1 ml of the cell culture solution and was incubated for 48 hours in a CO_2 incubator at 37°C . All incubations were performed in duplicate.

Table 2-9 A schematic of the plating technique used for the cell culture experiments

Unstimulated cells	Unstimulated cells + 1 μM 6-MP	Unstimulated cells + 0.5 μM 6-MP	Unstimulated cells + 0.25 μM 6-MP
BLANK CULTURE MEDIA	Unstimulated cells + 1 μM MTX	Unstimulated cells + 0.5 μM MTX	Unstimulated cells + 0.25 μM MTX
LPS Stimulated Cells	Stimulated cells + 1 μM 6-MP	Stimulated cells + 0.5 μM 6-MP	Stimulated cells + 0.25 μM 6-MP
EMPTY	Stimulated cells + 1 μM MTX	Stimulated cells + 0.5 μM MTX	Stimulated cells + 0.25 μM MTX

After 24 h of incubation, the supernatant was removed and stored at -8°C in labeled eppendorf tubes. New culture medium was added. At the end of 48 h, the adherent cells were re suspended. Cell viability was checked by trypan blue staining and cell count was performed for each well. The cell count was approximately 1×10^6 cells per ml. As expected the cells were a mixture of monocytes and lymphocytes.

The solution was then transferred to an eppendorf tube and centrifuged for 30 sat 12,000 rpm. The supernatant was divided into 4 eppendorf tubes so that repeated freeze thaw cycles could be avoided and the samples were frozen.

2.4.4 Enzyme linked immunosorbent assay (ELISA)

The antigen, in saline, is incubated on a plastic tray and a proportion adheres to the surface. This often requires overnight preparation. The free and surplus antigen is then washed away so that the surplus will not cause non specific binding. The test antibody is then added which will bind to the specific antigen and once again the unbound proteins are washed away. Subsequently, a ligand specific for the antibody attached to a peroxidase enzyme is added. After binding, the surplus is washed away and a chromogen is added that will provide a coloured product. The intensity of the colour is proportional to the amount of the antibody and relative differences are estimated by optical density scanning of the plate.

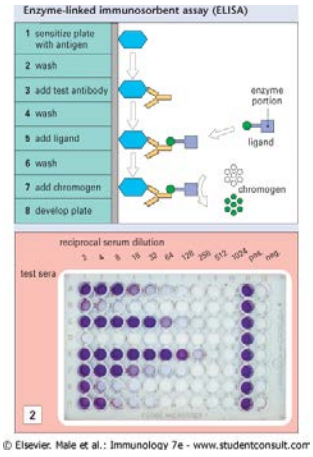


Figure 2-14 The technique of ELISA

[480]

2.4.4.1 Interleukin-10 (IL-10)

The IL-10 assay was performed with Human IL-10 ELISA Ready-set-go from eBioscience. (Catalog no: 88-7106) Sensitivity 2pg/ml, Standard curve ranges 2-300 pg/ml.
The kit contained: Pre titrated capture antibody (clone JES3-9D7), Pre titrated Biotin conjugated detection antibody (clone JES3-12GB), Standards (recombinant cytokine), ELISA coating Buffer powder, Assay Diluent: 5×concentration, Detection enzyme: pre titrated avidin-HRP, Substrate solution: Tetramethylbenzidine and a 96 Well Corning Costar 9018 plate.

Procedure: The plate was coated with 100µl of capture antibody diluted in the coating buffer and incubated overnight at 4°C. After repeated washing (5 times) with a well wash buffer, it was blotted on absorbent paper. The wells were then blocked with 200µl /well of assay diluent, incubated for 1 hour and then washed again. The standards were made up as described in the enclosed certificate of analysis. The 100 µl of the samples in duplicate were added to each well and incubated for 2 hours. Following this, 5 washes were performed and 100µl of detection antibody was added. The plates were washed again and 100µl Avidin-HRP was added. After further washes, 100µl of the substrate solution was added to each well, incubated for 15 minutes followed by 50µl of the stop solution. The plate was read at 450nM.

2.4.4.2 Soluble HLA-G (sHLA-G)

The soluble HLA-G assay was performed with an assay kit from Exbio, Praha, a.s (product no: RD194070100R) and measures sHLA-G1 and HLA-G5. The detection limits were 3 U/ml.
The kit contained Antibody coated microliter strips, Conjugate solution, Master calibrator, Dilution buffer, Wash solution (10×), Substrate solution and a Stop solution.

Procedure: The standards and the samples were loaded into the wells in duplicate and incubated for 16 h. Following this, the wells were washed (5×) and blotted on absorbent paper. The conjugate solution was added (100µl) was added to each well and incubated at room temperature for 1 h on an orbital microplate shaker. After further serial wash (5×) and drying by blotting, 100µl of the substrate solution was added and incubated at room temperature for 25 min. This is followed by the stop solution and the optical density was read with the setting at 450nm.

2.4.5 Measurement of other cytokine levels

Of the samples from individuals analysed in the previous study, 3 with a HLA-G del/del genotype and 3 with a HLA-G insertion allele were subsequently investigated for other cytokine levels. A dose effect was not being examined here; hence the doses of immunomodulators associated with the highest levels of sHLA-G were selected. The samples were analysed by Flowcytomix assay as described below.

2.4.5.1 Flow cytomix^{TM} assay

Fluorescent polystyrol beads are coupled with antibodies specific to the antigens to be detected. The required antibody linked beads are incubated with the samples to be tested. Specific binding takes place to the antibodies coupled to the beads. Following this, a biotin conjugated antibody mix is added, which binds to the bound capture antibodies. Finally, Streptavidin-Phycoerythrin (PE) is added which binds to the biotin conjugates. Two sets of beads with different sizes (4 μm & 5 μm) are used in the assay and each of the two sizes has different intensities of an internally fluorescent dye. The dye can be excited with a laser and the beads can be identified and quantified by flow cytometry.

The assay was utilized for the quantification of IL-10, IL-23, IL-8, IL-6, IL-18, IL-1β and TNF-α. The kit includes 1 vial set up beads, 1 bottle (10×) assay buffer (PBS with 10% BSA), 1 bottle reagent dilution buffer, 1 vial (200μl) Streptavidin-Phycoerythrin (Strepatvidin-PE) and a 96 well plate. In addition, each individual bead assay comprised of 1 vial of fluorescent beads (20×) coated with a specific antibody, Vial of lyophilized standard and a 1,350μl vial of Biotin-Conjugate (20×).
The proprietary software programme FlowCytomix Pro software provides a calculation of the customized volumes based on the number of samples and the number of assays being performed. Following this, the standards are reconstituted with all the standards from the intended assays. A serial dilution is performed as instructed.

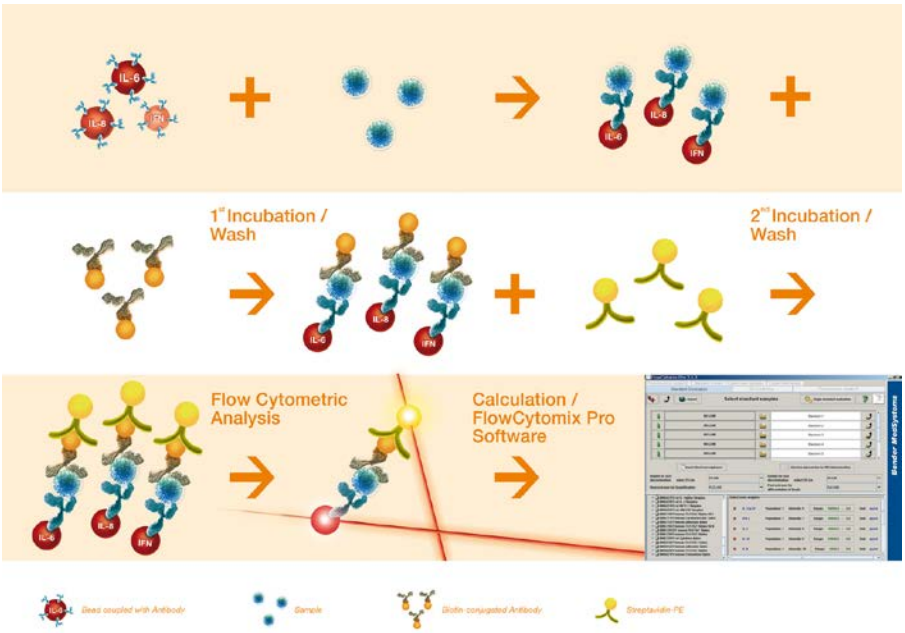


Figure reproduced from: [479]

Figure 2-15 Diagrammatic representation of the Flowcytomix assay process

The bead mixture is then prepared in a centrifugation tube. Each individual bead mix is vortexed, 1/20 of the calculated final volume is transferred into a vial and the volume was made up with reagent dilution buffer. This was then centrifuged at 3000×g for 5 min. All but 50µl of the buffer is removed and new buffer is added and the beads are resuspended by vortexing. The biotin-conjugate mixture is prepared and made up to volume with the supplied reagent dilution buffer. The supplied filter plate was pre moistened with 50µl of the assay buffer and aspirated by the vacuum manifold. Then 25µl of the standards were added in duplicate. An extra standard of the top concentration was added as instructed. Subsequently, the following sequential steps were performed 25 µl assay buffer, 25 µl samples, 25 µl bead mixtures and 50 µl Biotin-Conjugate mixtures.

The plate was protected from light by tin foil and incubated for 2 h on a microplate shaker at 500 rpm. This was followed by vacuum filtration of all the contents. The vacuum filtration was repeated twice with 100µl of assay buffer. A further 100µl of assay buffer was added along with

50µl of streptavidin-PE. The light free incubation process was repeated for a further 1 h on a microplate shaker. The contents of the wells were emptied by vacuum filtration after the addition of 100µl of the assay Buffer. This was repeated and then finally 200µl was added to each well, the contents transferred into a sample acquisition tubes for flow cytometry. The flow cytometer was set up with the supplied flow cytometry beads in accordance with the enclosed instructions.

The results were calculated with the proprietary Flowcytomix Pro 2.2 (Cat No BMS8400FF) software programme.

2.5 Statistics

Statistical analysis was performed with Prism 4 for Windows, version 4.03, GraphPad Software, San Diego California USA, www.graphpad.com.

Clinical response was correlated to genotype and a two sided Fisher's exact test was used to test for statistical significance. Dominant and recessive models were studied. In the dominant model both the homozygous mutants and the heterozygous individuals are affected. In the recessive model, only the homozygotes show an effect.

Statistical analysis for allele frequencies and haplotypes was performed using UNPHASED to test for association between clinical response / control status , side effects / control status, folate status and SNPs. PLINK was used for quality control for SNPs. Markers were analysed as single polymorphisms and in two-locus haplotypes within each gene.

The Chi-square test was used to calculate a deviation from Hardy-Weinberg equilibrium Hardy Weinberg equilibrium was calculated. The principle of the Hardy-Weinberg equilibrium describes the distribution of allele and genotype frequencies in a random population. It is expressed as $\mathbf{p^2 + 2pq + q^2 = 1}$. Where p is allele 1 and q is allele 2.

The frequency of allele 2 is

$$\frac{2\times (\text{the no. of 2/2 homozygotes}) + (\text{the no. of ½ heterozygotes})}{\text{The total no. of genotyped individuals}\times 2}$$

The frequency of allele 1 is p =1.0 - q

This formula is used to calculate the expected distribution of genotypes and the difference between the observed frequency and the expected frequency can be calculated with the chi ^{2} test ($\chi^2 = [o-e]^2 / e$). Deviation from this equilibrium is seen in abnormally distributed populations such has an inbred population, skewed allelic transmission or population stratification. However, the most common situation is genotyping error. The court lab HW calculator from www.tufts.edu was used for the calculations.

Gaussian distribution could not be assumed, hence, the non-parametric Mann-Whitney U test was used to test for an effect by genotype on the median change in sHLA-G and *IL-10* values after cell culture with the studied drug.

The standard unpaired t test measures the difference between means in two populations if the variance is equal. If the variance (namely the standard Deviation) is significantly different (measured by the Welch test as reported as a p value), there are some options to be considered.

- Reject the hypothesis and conclude the populations are different.
- Ignore the result. If the sample sizes are equal or nearly equal, the assumption of equal standard deviations is not crucial and the t test is robust. This is not a reliable approach if sample sizes are very small.
- Transform the data to log values. This should be planned in the study design.
- Run the t test with Welch correction for unequal variance. This has to be specified as part of the experimental design, otherwise there is risk of a Type I error. This is the option chosen in this study.

The Mann Whitney U test as a non parametric test does not provide a solution as it is also affected by unequal variance and does not test for variance

The two way ANOVA for repeated measures was used to test for significance in differences in cytokine levels and sHLA-G expression between samples incubated with and without the drug.

A p value less than 0.05 was taken to indicate statistical significance for all analysis. The contentious issue of how to adjust for multiple statistical tests is sometimes resolved by the application of the sequential Bonferroni correction. However, it is increasingly appreciated that there is a strong case for rejecting the use of this process when low p values are obtained.

Researchers could use the standard $p<0.05$ cutoff and make interpretations that would use principles recognized by reviewers such as study design, control groups selection, and basic logic. Relatively large p values in a large data table may not be relevant but would identify areas for examination. Clearly, truly negative results will not be replicated. Equally, small but significant effects will be identified [397]. Hence, the Bonferroni correction has not been applied in the statistical analyses reported in this work.

This work has a pragmatic design to reflect “real-world” clinical practice. MTX by dint of its role as second line therapy in CD is not widely used and access to DNA form well defined patient groups is relatively difficult. Hence, *a priori* power calculations for the experiments were not performed. Instead all available DNA as long as the patient phenotype was well defined and clinical information was available, was included. *Post hoc* power is not often useful and was not calculated in this thesis. The confidence intervals are all small particularly in the negative results and this can help identify genuine effects. The results of these experiments can help inform future power calculations for a prospective replication study.

3 Pharmacogenetic influence of polymorphisms in the MTHFR genes on clinical outcome of methotrexate therapy in patients with Inflammatory Bowel Disease

3.1 Introduction

A major focus of the work presented in this thesis is the examination of well recognised but inconsistent pharmacogenetic influences on methotrexate efficacy and toxicity in IBD patients. Of these, MTHFR polymorphisms are the most investigated in the folate metabolism pathway.

3.2 Aims

In this chapter, the influence of the two most frequent MTHFR genetic variants on individual response to therapy and the incidence of side effects are examined. Secondly, the effect of the effect of the MTHFR 677C>T and 1298A>C haplotypes on clinical response and side effects rates were studied. Thirdly, the influence of folate supplementation on these effects was studied.

3.3 Methods

3.3.1 Study design

Patients were recruited retrospectively from three tertiary referral centres in the United Kingdom. These included Guys and St Thomas's Hospital in London, Addenbrooke's Hospital, Cambridge and the John Radcliffe Hospital, Oxford. A significant proportion of these patients have formed part of two previously published studies concerning clinical outcome on MTX [398, 399]. Clinical details were collected by case note review by four independent assessors. Response was defined by a physician's clinical assessment as disease remission without concomitant steroid therapy after 3 months of treatment. Where MTX was used for fistula healing, response was defined as complete healing without discharge. Recourse to steroid therapy, Infliximab or surgery was, by definition, considered therapeutic failure. Adverse effects were defined by symptoms reported to the treating physician at clinic visits and review of laboratory investigations. Data collection of adverse events was not standardised as would be in a prospective trial. In most instances, such information was obtained from notes maintained by IBD nurse specialists.

Collection of data on folate supplementation was not universal across the three sites. Where such detail was available, the 5mg per day dose was the one used. The practice at the three centres that provided patients for the study was to use oral folates on all days of the week other than the day on which MTX was taken.

3.3.2 Laboratory methods

All patients were genotyped by Taqman SNP assay. The assay reliability was confirmed by RFLP in 25 patients. The correlation was 100% (Sections: 2.3.3 and 2.3.4).

3.3.3 Analysis

Statistical analysis for allele frequencies and haplotype was performed using UNPHASED to test for association between clinical response / control status , side effects / control status, folate status and SNPs. PLINK was used for quality control for SNPs. Markers were analysed as single polymorphisms and in two-locus haplotypes within each gene. Across genes, specific tests of association were performed. Statistical analysis of association between genotype frequencies and clinical response and side effects was performed with Prism 4 for Windows, version 4.03, GraphPad Software, San Diego California USA, www.graphpad.com. Clinical response was correlated to genotype and a two sided Fisher's exact test was used to test for statistical significance. Dominant and recessive models were studied. No correction for multiple testing within or across genes has been applied.

3.4 Results

A total of 201 patients were included in the study. Of these, 116 were female (57%). CD affected 145 (72%) patients and 38 (19%) had UC. 18 (8%) patients were classified as indeterminate colitis or data was unavailable. In the CD group, 40 (27%) had isolated colonic involvement, 75 (52%) had ileo-colonic involvement, 20 (14%) had isolated ileal involvement and 10 (6 %) had upper intestinal involvement. Fistulising perianal disease affected 41 (29%) patients. In the UC group 18 (47%) had left-sided colitis, 15 (39%) had pan colitis and five (13%) had isolated

proctitis. The mean age at diagnosis in the CD group was 29 years (range 9 to 78 years). In the UC group, the average age was 38 years, with a range of 19 to 75.

MTX was commenced as a steroid sparing agent in 111 (55%) of patients. There were 75 patients (44 %) who were not on steroids at the time of commencing MTX. Some of these individuals had received steroids in the past and had responded to treatment. A further 15 patients were resistant to steroids. MTX was used for failure of AZA to achieve a therapeutic response in 90 (48 %) patients and for AZA intolerance in 74 (36%) patients. AZA was not used in 11 patients and 10 patients decided to stop AZA for personal reasons. Data on prior AZA use was not available in the remainder. MTX was administered orally in 174 (86%) patients, subcutaneously in three patients and an initial intramuscular induction progressing to an oral regime in 22 (10%) patients. There was no difference in the response to treatment between the oral (87/179) and IM (9/22) regimes (p= 0.65 for response, OR = 1.366, CI= 0.5 – 3.3). A small number of patients (n=11) were treated with a combination of AZA and MTX of whom one patient had developed significant neutropenia, requiring cessation of MTX. 179 patients completed at least three months of MTX therapy and were included in the group for analysis of treatment efficacy. Response, defined as successful withdrawal of steroids in the steroid dependent group or the clear documentation of treatment efficacy in those who were not on steroids was noted in 97 patients. The Crohn's Disease Activity Index (CDAI) was not used and the Harvey Bradshaw Index (HBI) was not consistently documented. The mean dosage of MTX used was 20.34 mg/week (range 7.5 to 25 mg/week). There was no significant effect on clinical response of dosage (p=0.57), mode of administration (p=0.65) or folate supplementation (p=0.1527). Surgery was required in 85 patients. This included 76 patients with CD, 1 with indeterminate colitis and 7 with UC.

Side effects were noted in 49 patients. Gastrointestinal side effects were the most common with nausea or diarrhoea reported in 19 patients. Other side effects in decreasing order of frequency were transient LFT abnormalities in 12, haematological abnormalities including neutropenia in four, muscle and joint pains in four, minor infections in four, skin rash in three, mucositis in two and alopecia in one. The side effects were severe enough to lead to drug withdrawal in 17 patients. These included 5 for LFT abnormalities, 6 for GI intolerance, 1 for musculoskeletal symptoms, 1 for mucositis and 1 for impotence. Information on concurrent folate supplementation was available in 181 patients. Of these 114 received folate supplements and 67

patients did not. Folate supplementation (p=1.0) and MTX dosage did not influence side effect rates (p=0.08).

3.4.1 Genotyping results

The frequencies of the distribution of the homozygous mutation are displayed (Table 3-1)
The MTHFR677 and MTHFR1298 haplotype are in linkage disequilibrium (r^2= 0.05). The MTHFR 677T allele was more common among responders to MTX therapy. This finding just fails to meet statistical significance (p= 0.06, OR = 1.525, CI 0.912 - 2.394). Furthermore, we see that the major contribution to this effect comes from those who received oral folate (p= 0.01, OR = 2.241, CI = 1.19 – 4.2) and the effect was not seen in those individuals not receiving oral folate (p=0.924, OR = 1.038, CI = 0.478 – 2.251) (Table 3-2). More individuals with the *MTHFR677TT* genotype responded to therapy (14/19) and when compared with the wild-type *MTHFR677CC* genotype (40/86), this effect achieved significance for clinical response as a recessive effect (p=0.04, OR=3.2, CI= 1.06- 9.7) (Table 3-3)

In contrast, the MTHFR 1298C allele was not distributed significantly differently between responders and non responders to MTX therapy (p= 0.95, OR=1.0, CI= 0.6 – 1.53). Folate supplementation did not influence this effect (p= 0.1 for non- folate supplemented and p= 0.69 for folate supplemented) (Table 3-2)

Neither of the two polymorphisms influenced side effect rates and the mutant alleles were not distributed differently between those that experienced side effects and those that did not. The effects were also not influenced by folate supplementation (Table 3-4).

Compound heterozygosity with or without oral folate supplementation did not influence clinical response or side effect rates. Common haplotypes did not influence clinical response rates or side effects but the *677TT/1298AA* haplotype was seen more often in those who responded to therapy than in those who did not (p=0.03, OR= 1.6, CI = 1.2 – 2.3). The numbers in the groups were not large enough to allow further analysis of the effect of folate status (Table 3-5).

Table 3-1 MTHFR SNP frequency information

The frequencies reported are of the homozygous mutation.

SNP	rs no	Frequency in this study
MTHFR1298A>C	1801131	0.24
MTHFR677C>T	1801133	0.1

Table 3-2 The allele frequencies of the examined MTHFR allelotypes and their distribution between clinical responders and non responders.

In each case, the mutant allele is the allele for which the frequency, estimated Odds Ratio and p value refers. Table A refers to all cases (n-179), Table B to those who did not have oral folate (n=58) and Table C to those who did (n=103).

TABLE A

Gene	**All patients**				
	Response	**Non Response**	**OR**	**CI**	**p value**
MTHFR 677	0.357	0.267	1.525	0.971-2.394	0.065
MTHFR 1298	0.423	0.4205	1.011	0.664 - 1.538	0.959

TABLE B

Gene	**No folate supplements**				
	Response	**Non response**	**OR**	**CI**	**P value**
MTHFR 677	0.348	0.342	1.038	0.478 - 2.251	0.924
MTHFR 1298	0.484	0.34	1.827	0.855 - 3.901	0.116

TABLE C

Gene	**Folate supplemented**				
	Response	**Non response**	**OR**	**CI**	**p value**
MTHFR 677	0.368	0.206	2.241	1.191 - 4.217	0.01
MTHFR 1298	0.386	0.413	0.893	0.509 - 1.565	0.693

Table 3-3 The Recessive influence of the polymorphisms on the response to MTX therapy in patients with IBD

	Genotype	Clinical Response	Non Response	
MTHFR 1298A>C	AA	30	26	p=1, OR= 0.9 (0.4-2)
	AC	45	50	
	CC	16	12	
MTHFR 677C>T	CC	40	46	p=0.04, OR=3.2 (1.06- 9.7)
	CT	37	37	
	TT	14	5	

Table 3-4 The allele frequencies of the examined MTHFR allelotypes and their distribution between those that experienced side effects and those that did not

In each case, the mutant allele is the allele for which the frequency, estimated Odds Ratio and p value refers. Table A refers to all cases (n-201), Table B to those who did not have oral folate (n=66) and Table C to those who did (n=114).

TABLE A

Gene	All Cases				
	Side-effects	No side effects	OR	CI	p value
MTHFR 677	0.316	0.332	0.932	0.570 - 1.515	0.77
MTHFR 1298	0.438	0.427	1.046	0.663 - 1.654	0.846

TABLE B

Gene	No folate supplementation				
	Side-effects	No side effects	OR	CI	p value
MTHFR 677	0.366	0.372	0.975	0.419 - 2.268	0.953
MTHFR 1298	0.5	0.421	1.372	0.606 - 3.104	0.447

TABLE C

Gene	Folate supplementation				
	Side-effects	No side effects	OR	CI	p value
MTHFR 677	0.296	0.314	0.935	0.4804 - 1.822	0.844
MTHFR 1298	0.388	0.419	0.88	0.471 - 1.64	0.688

Table 3-5 The prevalence of the various MTHFR haplotypes in the study population and the influence of the haplotypes on clinical response

MTHFR Haplotype							
677	1298	Total	Clinical Response	Non Response	p (v/s all other haplotypes)	RR	CI
CT	AA	19 (10%)	12	7	0.2	NS	
TT	AA	16 (8%)	12	4	0.03	1.6	1.2 - 2.3
CC	AC	39 (20%)	18	21	0.8	NS	
CC	AA	25 (12%)	8	17	0.1	NS	
CC	CC	27 (14%)	15	12	0.4	NS	
CT	AC	69 (35%)	27	42	0.1	NS	

3.5 Discussion

Following on from the experience in cancer pharmacogenetics, the pharmacogenetic model has been applied to the investigation of side effects and clinical response rates in methotrexate therapy in RA and was initially focussed on the recognised polymorphisms in MTHFR.

As discussed earlier, the mutant homozygous *MTHFR 677TT* carriers exhibit 30% of the activity of MTHFR expected of a wild type and the heterozygote genotype is associated with 60% of wild type activity [370, 371] due to thermoloability. The 1298A>C SNP causes impaired activity that is relatively mild and does not influence plasma homocysteine levels in isolation. High folate states as seen in folate supplementation influences the stability of the mutant thermolabile enzyme making it more stable [375]. In folate deficient states, homozygous mutant *MTHFR 677TT* carriers show deficient DNA methylation.

Interestingly, in this study, the 677C>T mutation did not influence side effects and folate supplementation did not appear to change this. This is not completely at odds with previous reports as has been described in my review of the literature. Although, it has been associated with increased side effect rates, a number of reports have not found any associations [400]. The

677C>T mutation causes homocysteinemia in some individuals exposed to MTX. There is relative depletion of the folate pool in the form of 5′- methyl THF that would otherwise have been generated from 5′, 10′ methylene THF. The subsequent remethylation of methionine from homocysteine is impaired and there is a relative homocysteinemia. Serum homocysteine levels rise in 24 hours after MTX administration and remain elevated for up to 7 days [401]. In MTX therapy of ovarian malignancies, the mutation has a dose effect with greater homocysteine levels seen in the *MTHFR 677TT* genotype when compared to heterozygotes that have higher levels than the wild type genotypes [402]. This may predispose to toxicity and associations have been made with hepatotoxicity, haematological toxicity, mucositis and neurotoxicity. The homocysteinemia is seen in both cancer chemotherapy [403] and anti inflammatory therapy [402]. However, in RA the effect although clear and very obviously abolished by oral folate supplementation, was not related to the 677C>T mutation [404]. This finding is predominantly described at higher MTX doses [405] and may explain the lack of an association in this study where much lower doses were used.

This data shows that the *MTHFR 677TT* genotype is associated with a greater chance of clinical response to MTX therapy (p=0.04, OR=3.2 (1.06-9.7) as a recessive effect. Furthermore we note that folate supplementation appears to play a role. All those individuals with the *MTHFR 677TT* mutant genotype receiving oral folate, responded to therapy (n=10). Among those who did not receive oral folate supplements, 5/11 (45%) failed treatment (p=0.03, OR = 17). There are more individuals with the *MTHFR1298AA* genotype in this group and it is possible that the *MTHFR 677TT/1298AA* haplotype is exerting a previously unrecognised influence (p=0.03, OR= 1.6, CI = 1.2 – 2.3). As stated earlier the group was too small for sub group analysis into the effect of folate supplementation and a hypothesis to explain such an effect is lacking. A larger study population may throw up some answers.

In cancer pharmacogenetics, there appears to be an effect specific to cell type for the 677C>T genotype on response to MTX. Breast cancer cell lines are less responsive *in vitro* than colon cancer cells. [406]. In Acute Lymphocytic Leukaemia (ALL) *ex vivo* studies have been contradictory with both positive and negative effects on drug response attributed to the 677C>T polymorphism [407, 408]. In clinical studies on leukaemia patients, 677C>T has a negative effect on clinical response in most reports [409]. In RA, once again results are conflicting. Two studies showed a negative effect of 677C>T polymorphism on response [392, 410] and one showed a positive response [411]. In IBD and Psoriatic arthritis, no effects of this polymorphism were

noted on clinical response [399, 412]. The higher plasma levels of MTX seen in patients with the *MTHFR 677TT* genotype in a report on Japanese patients with acute myeloid leukaemia (AML) may be very relevant to the findings. In this small study, individuals with the *MTHFR 677TT* genotype had high serum MTX levels that were toxic enough for leucovorin rescue [405]. As described earlier concurrent folate therapy appears to reduce the homocysteinemia and certainly increases drug tolerance in the *MTHFR 677TT* group [375]. While a coherent reason for MTHFR influencing MTX serum levels cannot be made at this time, this observation may explain the effect of *MTHFR 677TT* on clinical response seen in this study. Other reasons for a similar effect may be that reduced methionine and s-adenosyl methionine lead to impaired availability of a methyl group for DNA methylation and may augment MTX immunosuppression. Furthermore, MTX induced inhibition of TS, when associated with impaired MTHFR activity reduces the availability of THF derivatives that may increase the effects of immunosuppression [411]. Importantly in the absence of folate supplementation, these effects would be expected to cause systemic toxicity [411]. The MTHFR 1298A>C polymorphism has been investigated in paediatric ALL and the mutant genotype appears to predispose to a poorer clinical response; one that is unusually not seen in either homozygote genotypes [408]. In RA, positive [413-415] and negative [410] effects of MTHFR 1298A>C on clinical response have been noted. No effects have been seen in IBD and psoriasis [399, 412]. This polymorphism has no effect on efficacy or side effects to MTX therapy and that folate supplementation does not appear to influence this effect. There is no influence of compound heterozygosity on clinical response or side effect rates of MTX. Hence the hypothesis that *MTHFR 1298AC* or the *MTHFR 1298CC* genotype would demonstrate a compromised clinical response in comparison to the wild type genotype or that this effect would be diminished by folate supplementation was not supported. The hypothesis that the *MTHFR 677TT* genotypes would have a poorer clinical response than the other genotypes was not supported but instead, the co-administration of oral folate appeared to improve the clinical response in this group. This effect could be attributed to the effects of high folate states on enzymatic stability. The hypothesis that the mutations would influence side effect rates or that the compound heterozygosity would influence clinical response or side effect rates were not supported by the data. Furthermore, oral folate supplementation did not influence these effects.

Clearly the influence of pharmacogenetic variation on other folate pathway enzymes may be important and this is examined in the next chapter.

4 The effect of polymorphisms in the thymidylate synthase gene and other non-synonymous polymorphisms on response to methotrexate in IBD

4.1 Introduction:

A number of other polymorphisms have been examined, predominantly in the analysis of clinical response and side effect rates of individuals receiving cancer chemotherapy. Replication has then been attempted in RA and psoriasis with limited and often variable success. Thymidylate synthase is inhibited directly by polyglutamated MTX. A tandem repeat sequence of 28 base pairs within the 5′ untranslated region (UTR) of the TS gene can contain either 2 or 3 repeats. Homozygosity for the triple repeat allele (*TSER *3R/*3R*) is associated with greater mRNA transcriptional activity that is manifest as enhanced enzymatic activity. This may decrease clinical response and not influence side effect rates. This effect would be abolished by folate supplementation. A six bp deletion (TTAAAG) at position 1494 in the 3′ UTR of this gene is associated with decreased mRNA stability and lower mRNA levels are noted in the homozygote carriers of the mutant deletion. Carriers may experience an enhanced therapeutic benefit with MTX. These two polymorphisms may be in LD. The 3*R with the 6bp insertion has greater transcriptional efficiency and more mRNA stability than the 2R with the 6 bp deletion. MTHFR and TS are closely related in the maintenance of THF and single carbon methyl groups which are essential for DNA methylation and possibly the epigenetic modification of cytokine genes. The *MTHFR677TT* mutation may impart reduced clinical efficacy on MTX therapy to individuals who possess the TS 2*R with the TS 6bp deletion.

A single nucleotide polymorphism in the SLC19A1_gene appears to lead to loss of SLC19A1 gene expression. *SLC19A1 80GG* may impair cellular influx of MTX preventing polyglutamation and impairing response to MTX therapy. *ATIC 347 GG* has been correlated with a better clinical response than *ATIC 347CC* and *ATIC 347CG*. Another group has reported the opposite effect. *AOX 3404AG* and the rare *AOX 3404GG* polymorphism may improve clinical response by reducing the synthesis of inefficacious 7-OH MTX derivatives.

4.2 Aims:

I. To correlate clinical response to MTX therapy with the *TSER3*R/3*R* and the *TS6bp ins/ins* genotypes and to examine the effect of folate supplementation on this correlation. The hypothesis is that the mutant homozygote polymorphisms would be associated with decreased clinical response and that oral folate supplements would abolish this effect. Either of these polymorphisms would not be expected to influence side effect rates and folate supplementation would not be expected to play a role.

II. To examine the effect of the combined TSER 3*R and TS 6bp ins on clinical response. It is hypothesised to be higher than the adverse genotypes and that folate supplementation would not influence these results. Side effect rates will not be affected.

III. To evaluate the effect of the *MTHFR 677TT* on individuals with the TSER2*R and the TS6bp del. It may be impaired and oral folate supplementation may reverse this effect.

IV. To examine the effect of the *SLC19A1 80GG* mutation with on clinical response and side effect rates to MTX with and without folate supplementation. The polymorphisms may impair clinical response and folate supplements would have no effect.

V. To study the influence of ATIC 347C>G polymorphisms on clinical response rates and side effect rates to MTX in the study group. Oral folate supplementation is unlikely to have an effect.

VI. To examine the effect of the AOX 3404A>G polymorphisms on response to MTX therapy. Folate supplementation may play a role as the folate status can affect cellular efflux of MTX. As SLC19A1 is the major efflux channel, the *SLC19A1 80GG* mutation may abolish this effect. All these influences will be evaluated

4.3 Methods

Criterion for assessment of clinical response and side effects as well as the population used in this chapter was the same as that selected for the MTHFR study in the preceding chapter. The demographic and phenotype of the study group is represented in the table (Table 4-1 and Table 4-2).

A combination of allelic discrimination and Taqman SNP assay was used in this experiment. The SLC19A1 80A>G was genotyped by RFLP. The TS1494 6bp ins/del and the TSER 3*/2* repeat were genotyped by allelic discrimination by sizing assay. The AOX1 3404A>G, the MTHFR 1298A>C and the MTHFR 677C>T were genotyped by Taqman SNP assay. The validity of the Taqman assay for AOX1 3404A>G was confirmed by direct sequencing in our lab (results not displayed) and the MTHFR 1298A>C and MTHFR 677C>T by RFLP in a selected group of patients. Details of these techniques are available in Chapter 2 (page no 86 and 88).

Table 4-1 Characteristics and therapeutic details of the patients included in the MTX pharmacogenetics experiments

Total Patients			201
Females			116 (57%)
Disease type	Crohn's Disease	All cases	145 (72%)
		Colonic Disease	40 (27%)
		Ileo Colonic	75 (52%)
		Ileal	20 (14%)
		Upper Intestinal	10 (6%)
		Perianal	41 (29%)
	Ulcerative Colitis	All cases	38 (19%)
		Left sided disease	18 (47%)
		Pancolitis	15 (39%)
		Proctitis	5 (13%)
	Indeterminate colitis	All cases	18 (9%)
Age at diagnosis	Crohn's disease		29 (Range 9 - 78 years)
	Ulcerative Colitis		38 (Range 19 - 75 years)
Extra Intestinal disease	Crohn's Disease	Arthritis	39 (26%)
		Erythema Nodosum	6 (4%)
		Ocular disease	10 (6%)
	Ulcerative Colitis	Arthritis	7 (18%)
		Erythema Nodosum	1 (2%)
Indication for MTX	All patients	Steroid sparing	111 (55%)
		Steroid resistance	15 (7%)
		Azathioprine failure	74 (36%)
		Patient preference	21 (10%)
Route of Administration	All patients	Oral	174 (86%)
		Sub cutaneous	3 (1.5%)
		IM induction / oral maintenance	22 (10%)
Mean Dosage	All patients		2.34 mg (7.5 to 25 mg/wk)
Folate supplementation		Yes	114
		No	67
		Data Unavailable	20
Response to Therapy	179 Patients	Yes	97 (54%)
		No	82 (46%)
Recourse to surgery	CD		76 (38%)
	UC		7 (3%)
	Indeterminate		1

Table 4-2 Side effects affecting the MTX group leading to treatment cessation

Side effects	Number of individuals affected	Withdrawal from treatment
Gastrointestinal	19	6
Liver function tests	12	5
Haematological	4	1
Musculoskeletal	4	2
Skin rash	3	1
Mucositis	2	1
Alopecia	1	0
Minor Infections	4	0
Impotence	1	1
Total	50	17

Analysis

Statistical analysis for allele frequencies and haplotypes was performed as described in Chapter 2.

4.4 Results:

A total of 201 patients were included in the side effects group and 179 patients who completed therapy for 3 months were included in the clinical response group. The frequencies of the mutant homozygotes are displayed in (Table 4-4).

All the genotypes were in HWE. There were differences in the distribution of alleles between clinical responders and non responders when all patients were examined together. Although, overall, there was no significant difference in clinical response rates between folate supplemented and un-supplemented individuals, it was possible to discern differences in clinical response rates between those individuals that received oral folates and those that did not when stratified for certain genotypes.

The TSER 3* allele was present in 65 of 91 (71%) individuals who experienced a clinical response and in 68/88 (77%) of those who failed therapy (p=0.3, OR =1.3, CI 0.6 -2.6). The TS 1494del6 allele was present in 62 of the 91 clinical responders (68%) as compared with 71/88 (80%) non responders (p=0.06, OR =1.9, CI= 0.9 – 3.9).

In the recessive model, the *TSER 3*/3** did not have a significant correlation with clinical response (p=0.68, OR= 1.2, CI= 0.5 – 2.6). Of those who possessed the *TSER 3*/3** genotype, 20/46 experienced a clinical response (43%). In the recessive model, the homozygous *TS 6bp del/del* genotype was equally distributed among the clinical responders and non responders (49 % v/s 51%) (p=0.3, OR=0.6, CI= 0.2 – 1.3). (Table 4-6)

The high producer TS haplotype (TSER 3*/ TS 6bp ins) was equally distributed between clinical responders (43/91, 47%) and non-responders (45/88, 51%), [p= 0.65, OR = 1.16, CI= 0.64 -2.1]. (Table 4-6)

The SLC19A1 80G allele was present in 61% of clinical responders and 68% of non-responders (p=0.43, OR= 1.3, CI=0.7 – 2.48). The recessive effect of the *SLC19A1 80GG* was also not significant for clinical response (p=0.8 OR= 1.169, CI= 0.4-2.7).

The ATIC 347G allele was more common among responders to MTX therapy (p= 0.03, OR = 1.57, CI 1.02 -2.42). Furthermore, we see that the major contribution to this effect comes from those who did not receive oral folate. (p= 0.05, OR = 2.12, CI = 0.98 – 4.5) and the effect was not seen in those individuals receiving oral folate. More individuals with the *ATIC 347CC* genotype failed therapy (18/27) and when compared with the wild-type *ATIC 347GG* genotype, this effect just failed to achieve significance for clinical response as a recessive effect (p=0.07, OR=2.5 CI=1-6.458). The AOX 3404A>G polymorphism did not influence clinical response rates either in a dominant (p= 0.75) or recessive model (p=0.6) and allele frequencies did not differ between the clinical responders and non responders and folate supplementation did not appear too play a role. (Table 4-3, Table 4-5, Table 4-6)

Side effects

Overall side effect rates were low and MTX was well tolerated. In this relatively small group subgroup analysis was not possible and hence, side effects are presented as whole Only ATIC 347C>G correlated with side effect rates. Individuals with the C allele were more likely to be free of side effects if they were receiving folate supplementation (OR= 2.09, CI = 1.13 – 3.8, p = 0.01). (Table 4-9)

Haplotype analysis

The two tested TS polymorphisms have been reported to be in LD [416] and some have described a co existence of the high TS activity TSER3*R allele and the low TS activity TS6bp del allele. Others have not found this effect. In this study, they are not in LD ($D' = 0.06$). The proposed high activity haplotype did not influence clinical response rates or side effect rates with or without oral folate supplementation. (Table 4-8, Table 4-9). The MTHFR677/TS 3*R/TS6bp del haplotype did not influence side effects or clinical response and was not affected by folate supplementation. (Table 4-11)

For these haplotype models, we need to focus on the global p-value. The model also tests the effects of individual haplotypes, but this is a secondary analysis, to explore haplotype effects when the overall effect is significant. The only significant result comes from the clinical response, folate group (p=0.029), but this is driven by the MTHFR 677 variant which alone has a significant effect on clinical response in patients with folate. Further sub-models, testing whether either or both TS variants had a significant effect in addition to the MTHFR 677 effect, showed that the haplotype signal seen here comes entirely from MTHFR 677. No significant effects of the haplotype were seen in the non folate group or in the study cohort as a whole. The haplotype did not influence side effect rates with or without folate.

Table 4-3 The allele frequencies of the examined TS, ATIC, SLC19A1 AND AOX allelotypes and their distribution between clinical responders and non responders.

In each case, the mutant allele is the allele for which the frequency, estimated Odds Ratio and p value refers. Table A refers to all cases (n=179), Table B to those who did not have oral folate (n=58) and Table C to those who did (n=103).

TABLE A

Gene	**All patients**				
	Response	**Non Response**	**OR**	**CI**	**p value**
SLC19A1 80G	0.412	0.414	0.989	0.6493 - 1.506	0.958
ATIC 347G	0.423	0.318	1.571	1.02 - 2.422	0.039
AOX 3404G	0.159	0.159	1.002	0.568 - 1.765	0.994
TS 3*R	0.5	0.477	1.095	0.7235 - 1.658	0.667
TS 6bp Ins	0.511	0.448	1.283	0.8467 - 1.944	0.239

TABLE B

Gene	**No folate supplements**				
	Response	**Non response**	**OR**	**CI**	**p value**
SLC19A1 80G	0.53	0.38	1.842	0.871 - 3.893	0.106
ATIC 347G	0.5	0.32	2.125	0.988 - 4.569	0.0505
AOX 3404G	0.181	0.14	1.365	0.494 - 3.765	0.544
TS 3*R	0.515	0.42	1.467	0.699 - 3.077	0.308
TS 6bp Ins	0.4394	0.38	1.279	0.604 - 2.707	0.519

TABLE C

Gene	**Folate supplemented**				
	Response	**Non response**	**OR**	**CI**	**p value**
SLC19A1 80G	0.35	0.402	0.803	0.455 - 1.417	0.449
ATIC 347G	0.386	0.357	1.124	0.636 - 1.985	0.687
AOX 3404G	0.1491	0.163	0.899	0.422 - 1.916	0.784
TS 3*R	0.4825	0.51	0,8925	0.515 - 1.546	0.685
TS 6bp Ins	0.543	0.4239	1.62	0.931 - 2.819	0.086

Table 4-4 SNP frequency information for TS, ATIC, SLC19A1 and AOX

The frequencies reported are of the homozygous mutation.

SNP	Frequency in this study	HWE χ^2
TSER3*/2*	0.49	0.23
TS1494del6	0.52	1.94
ATIC 347C>G	0.37	0.09
SLC19A1-1 80A>G	0.42	0.06
AOX1 3404A>G	0.009	1.89

Table 4-5 The dominant effect of the studied TS, ATIC, SLC19A1 and AOX genotypes on response rated to MTX therapy

SNP	CR	NR	p	OR	CI
TSER3*/2*	65/91 (71%)	64/88 (72%)	0.3	1.3	0.6 - 2.6
TS1494del6	62/91 (68%)	71/88 (80%)	0.06	1.9	0.9 - 3.9
SLC19A1-1 80A>G	56/91 (61%)	60/88 (68%)	0.43	1.3	0.7 - 2.48
AOX1 3404A>G	29/91 (32%)	26/88 (30%)	0.75	1.11	0.5 - 2.1
ATIC 347C>G	59/91 (65%)	47/88 (53%)	0.13	0.6	0.3 – 1.1

CR- Clinical responders, NR- Non responders

Table 4-6 The distribution of the TS, SLC19A1, ATIC and AOX genotypes between clinical responders (CR) and non responders (NR) and the recessive effects of these polymorphisms on response rates

SNP	p	OR	CI
TSER3*/2*	0.3	0.73	0.37 - 1.4
TS1494del6	1	0.9	0.5 - 1.8
SLC19A1-1 80A>G	0.3	0.67	0.31 - 1.46
AOX1 3404A>G	0.75	1.11	0.5 - 2.1
ATIC 347C>G	0.07	2.5	1 - 6.4

Table 4-7 Haplotype analysis of the effect of the TYMS 5'UTR and 3'UTR Polymorphisms on the clinical response of IBD patients to MTX therapy

P=0.65, OR = 1.1, CI= 0.6 – 2.1 for the 3R/6bp ins haplotype compared with other genotypes for clinical response.

	Therapeutic failure			Clinical Response		
	6bp ins/ins	6bp ins/del	6bp del/del	6bp ins/ins	6bp ins/del	6bp del/del
2R/2R	3	14	7	7	14	5
2R/3R	11	20	13	16	11	12
3R/3R	3	11	6	6	10	10

Table 4-8 Influence of the TS 3*R and TS 6bp haplotype on side effect rates

	Side effects			Tolerated		
	6bp ins/ins	6bp ins/del	6bp del/del	6bp ins/ins	6bp ins/del	6bp del/del
2R/2R	2	6	2	10	24	10
2R/3R	**8**	**12**	10	**22**	**26**	18
3R/3R	**3**	**2**	4	**7**	**20**	14

Table 4-9 The allele frequencies of the examined TS, SLC19A1, ATIC and AOX allelotypes and their distribution between those that experienced side effects and those that did not.

Table A includes all the subjects (n=201), Table B are those that did not receive oral folate supplementation (n=66) and Table C are those that did (n=114). In each case, the mutant allele is the allele for which the frequency, estimated Odds Ratio and p value refers.

TABLE A

Gene	All Cases				
	Side-effects	No side effects	OR	CI	P value
SLC19A1 80A>G	0.438	0.411	1.121	0.706 - 1.773	0.63
ATIC 347C>G	0.438	0.352	1.439	0.905 - 2.288	0.124
AOX 3404A>G	0.163	0.144	1.153	0.617 - 2.151	0.657
TSER3*/2*	0.489	0.486	1.012	0.641 - 1.596	0.959
TS 6bp ins/del	0.458	0.486	0.891	0.562 - 1.413	0.625

TABLE B

Gene	No folate supplementation				
	Side-effects	No side effects	OR	CI	P value
SLC19A1 80A>G	0.4	0.48	0.721	0.315 - 1.649	0.436
ATIC 347C>G	0.3	0.441	0.542	0.226 - 1.3	0.161
AOX 3404A>G	0.166	0.147	1.161	0.384 - 3.504	0.794
TSER3*/2*	0.4	0.48	0.721	0.315 - 1.649	0.436
TS 6bp ins/del	0.4	0.431	0.878	0.383 - 2.013	0.759

TABLE C

Gene	Folate supplementation				
	Side-effects	No side effects	OR	CI	P value
SLC19A1 80A>G	0.425	0.362	1.307	0.702 - 2.434	0.4
ATIC 347C>G	0.518	0.339	2.099	1.13 - 3.899	0.0189
AOX 3404A>G	0.111	0.155	0.68	0.265 - 1.747	0.409
TSER3*/2*	0.537	0.488	1.215	0.658 - 2.24	0.533
TS 6bp ins/del	0.5	0.494	1.023	0.555 - 1.884	0.941

Table 4-10 The effect of the MTHFR 677/TS 3*R/TS 6bp ins haplotype on clinical response rates and the effect of folate supplementation in this group. The variant allele is each instance is accorded the number 1 (MTHFR677T, TS 3*R, TS 6bp Del). The wild type allele in each instance is 2. The reference haplotype is 1-1-1

All Cases								
Haplotype	Case	Control	Ca-Freq	Co-Freq	Odds-R	CI	P-value	Likelihood ratio
1-1-1	31.59	42.67	0.17	0.24	1.00	1	0.08	Chi sq=4.789, df=3, p=0.187
1-1-2	28.24	28.67	0.16	0.16	1.28	0.8 – 1.9	0.83	
1-2-1	26.31	29.85	0.14	0.17	1.08	0.7 – 1.6	0.46	
1-2-2	30.86	27.80	0.17	0.16	1.38	0.7 – 2.5	0.74	
2-1-1	8.89	6.67	0.05	0.04	1.51	0.9 - 2.3	0.52	
2-1-2	22.28	13.98	0.12	0.08	1.92	1 – 3.5	0.11	
2-2-1	22.21	17.81	0.12	0.10	1.63	0.8 - 3	0.47	
2-2-2	11.62	8.54	0.06	0.05	2.09	0.9 - 4	0.39	
Folate supplemented								
Haplotype	Case	Control	Ca-Freq	Co-Freq	Odds-R	CI	P-value	Likelihood ratio
1-1-1	13.48	20.35	0.12	0.22	1.00	1	0.03	Chi sq=9.04, df=3, p=0.02
1-1-2	22.64	14.71	0.20	0.16	1.58	0.8 – 2.8	0.41	
1-2-1	21.02	21.83	0.18	0.24	0.99	0.5 – 1.7	0.30	
1-2-2	14.86	16.1	0.13	0.18	1.57	0.6 – 3.5	0.32	
2-1-1	5.14	4.323	0.05	0.05	2.20	1 - 4	0.93	
2-1-2	17.75	5.614	0.16	0.06	3.46	1.4 - 8	0.01	
2-2-1	12.36	6.493	0.11	0.07	2.19	0.9 - 5	0.26	
2-2-2	6.75	2.571	0.06	0.03	3.44	1.2 - 9	0.13	
No Folate Supplementation								
Haplotype	Case	Control	Ca-Freq	Co-Freq	Odds-R	CI	P-value	Likelihood ratio
1-1-1	17.53	16.36	0.2655	0.3271	1	1	0.44	Chi sq=1.29, df=3, p=0.73
1-1-2	6.347	6.823	0.09617	0.1365	1.205	0.5 – 2.5	0.42	
1-2-1	5.338	4.89	0.08087	0.0978	1.448	0.6 - 3	0.72	
1-2-2	13.79	4.929	0.2089	0.09859	1.745	0.5 - 5	0.074	
2-1-1	3.867	2.589	0.05859	0.05179	0.9409	0.4 - 2	0.84	
2-1-2	4.26	3.23	0.06455	0.06461	1.134	0.3 – 3.4	0.99	
2-2-1	10.27	7.163	0.1556	0.1433	1.362	0.4 - 4	0.83	
2-2-2	4.603	4.017	0.06975	0.08034	1.642	0.4 - 6	0.77	

Table 4-11 The effect of the MTHFR 677/TS 3*R/TS 6bp ins haplotype on side effect rates and the effect of folate supplementation in this group
The variant allele is each instance is accorded the number 1 (MTHFR677T, TS 3*R, TS 6bp Del). The wild type allele in each instance is 2. The reference haplotype is 1-1-1. Ca- Freq (Frequency of cases), Co- freq (frequency of controls)

All Cases								
Haplotype	Case	Control	Ca-Freq	Co-Freq	Odds-R	CI	P-value	Likelihood ratio
1-1-1	19.82	59.18	0.21	0.19	1.00	1.00	0.77	Chi sq=0.26, df=3, p=0.96
1-1-2	14.24	51.03	0.15	0.17	0.89	0.5- 1.42	0.60	
1-2-1	15.28	46.36	0.16	0.15	1.01	0.63 - 1.60	0.85	
1-2-2	15.65	46.43	0.16	0.15	0.90	0.4 - 1.73	0.78	
2-1-1	5.34	14.97	0.06	0.05	0.96	0.58 - 1.57	0.71	
2-1-2	9.60	30.83	0.10	0.10	0.86	0.43 - 1.68	0.96	
2-2-1	11.56	35.49	0.12	0.12	0.97	0.49 - 1.90	0.91	
2-2-2	4.51	19.72	0.05	0.06	0.86	0.38 - 1.95	0.38	
Folate supplemented								
Haplotype	Case	Control	Ca-Freq	Co-Freq	Odds-R	CI	P-value	Likelihood ratio
1-1-1	8.17	28.45	0.15	0.16	1.00	1	0.80	Chi sq=0.46, df=3, p=0.92
1-1-2	8.84	33.07	0.16	0.19	1.07	0.5 - 1.99	0.60	
1-2-1	12.19	33.84	0.23	0.19	1.23	0.6 - 2.29	0.55	
1-2-2	8.81	24.64	0.16	0.14	1.31	0.5 - 3.17	0.63	
2-1-1	2.09	7.844	0.04	0.05	0.93	0.4 - 1.82	0.73	
2-1-2	5.91	19.64	0.11	0.11	1.00	0.39 - 2.49	0.93	
2-2-1	4.55	17.87	0.08	0.10	1.15	0.4 - 2.87	0.62	
2-2-2	3.45	8.649	0.06	0.05	1.23	0.4 - 3.71	0.53	
No Folate Supplementation								
Haplotype	Case	Control	Ca-Freq	Co-Freq	Odds-R	CI	P-value	Likelihood ratio
1-1-1	8.44	27.14	0.28	0.27	1.00	1	0.86	Chi sq=0.65, df=3, p=0.88
1-1-2	3.80	12.46	0.13	0.12	0.93	0.3 - 2.16	0.94	
1-2-1	2.54	8.588	0.08	0.08	0.72	0.3 - 1.71	0.99	
1-2-2	4.22	15.82	0.14	0.16	0.67	0.2 - 2.25	0.83	
2-1-1	3.03	7.813	0.10	0.08	1.05	0.4 - 2.51	0.60	
2-1-2	2.74	5.591	0.09	0.05	0.98	0.2 - 3.29	0.38	
2-2-1	3.99	14.46	0.13	0.14	0.76	0.2 - 2.59	0.89	
2-2-2	1.24	10.13	0.04	0.10	0.70	0.15 - 3.13	0.21	

4.5 Discussion:

4.5.1 Thymidylate synthase enhancer region (TSER) tandem repeats, TS 6bp insertion/deletion and MTHFR677 Polymorphisms

The hypothesis that the polymorphisms increasing TS function would correlate with an impaired response to MTX therapy is not supported by the evidence. Hence, predictably, folate supplementation does not have an effect on the association. TS competes with the methionine cycle for the substrate 5, 10-methylene tetrahydrofolate. When the TS activity is increased as may be seen in the case of the TSER 3*R and the TS 6bp insertion, the expected inhibition by antifolate therapy may not be seen. High folate states would supply both the methionine and the thymidylate pathways and homocysteine levels would be normal. Low folate states would demonstrate the opposite effect and the resulting homocysteinemia may influence side effect rates.

MTX therapy induces homocysteinemia that is abolished by oral folate or folinic acid supplementation [417]. The two formulations do not differ in their effects and there is no influence of the MTHFR 677 genotype on this effect. Other groups have reported that folic acid and folinic acid supplementation did not affect the homocysteine levels seen in MTX treated patients implying that the DHFR inhibition seen with MTX therapy may not be relevant in the low anti inflammatory doses used in this study. MTHFR is strategically positioned between the methionine and the thymidylate pathways. The methionine pathway as described earlier can be affected by variant MTHFR activity and may have some impact on the clinical efficacy of antifolate therapy such as MTX. This may explain the positive association of the *MTHFR 677TT* genotype on clinical response in Chapter 3 (page 114). The haplotype analyses here do not support an interaction between MTHFR and TS on clinical response to MTX.

There could be a number of reasons for this. In addition to body folate levels, Vitamin B12 and Vitamin B6 are important co-factors and may have confounded the effect of the studied polymorphisms. Deficiencies of Vitamin B12 can result in increases in plasma homocysteine levels [418]. There is a G/C functional SNP in the second repeat of the 3*R allele that appears to influence clinical response to oral fluoropyrimidine adjuvant therapy. The G allele is proposed to decrease TS transcription and reduce clinical response to therapy [419] and the combination of

the three TS high activity polymorphisms are additive on serum homocysteine levels [420]. Data on this polymorphism are not available in this study.

4.5.2 The Reduced Folate carrier (SLC19A1)

The hypothesis that individuals with the *SLC19A1 80AA* genotype would be predisposed to an improved outcome from MTX therapy in this study of individuals with IBD could not be proven. We have also found that the polymorphism did not influence side effect rates. As was expected, oral folate supplementation did not affect these findings. The elevated MTX levels seen in leukaemia in carriers of the *SLC19A1 80AA* genotype [421] and higher methotrexate polyglutamate levels and improved therapeutic benefit in RA [338, 422, 423] has been at odds with the results of a large prospective trial in RA [422] and a retrospective study in IBD [399]. A retrospective study in psoriasis associated the SLC19A1 80A with side effects but not with response to medication. Importantly, in functional studies, no differences were noted in the uptake of MTX by leukaemic blast cells with the mutant SLC19A1 protein versus the wild type [424] and the role of this polymorphism in modifying MTX drug response remains controversial.

SLC19A1 is considered to be a relatively low capacity transporter of folate and anti folates [294] in comparison to the folate binding protein (FBP) or the folate receptor (FR) which has higher affinity for reduced folate cofactors and folic acid relative to the antifolates. This mechanism maintains cellular folate levels [295]. While the low pH transporter, expressed independently of SLC19A1 transports reduced folates and anti folates such as MTX, its relevance as the transporter mechanism at physiological pH is questionable [425, 426]. Despite that, these alternative routes of folate transport may be critical in tissues with low SLC19A1 expression and may explain the lack of correlation in this experiment.

4.5.3 ATIC

That adenosine is an important mediator of the anti-inflammatory effects of MTX is not contested. MTX inhibits ATIC and this may lead to an intracellular accumulation of adenosine. This occurs by way of accumulation of AICAR. This compound inhibits the catabolism of both AMP and adenosine by the inhibition of the enzymes AMP deaminase and ADA. The intracellular adenosine accumulation is followed by extracellular efflux. These findings have not been restricted to RA. In IBD, a similarly pro-inflammatory state that is attenuated by ADA

inhibitors has been described in an animal model [427]. Adenosine inhibits neutrophilic recruitment, adhesion and the generation of superoxide anions [428]. In experimental colitis, it attenuates mucosal TNF-α, IFN-γ and plasma TNF-α, and IL-6 [427]. The anti-inflammatory IL-10 and TGF-β are relatively unaffected [429]. In essence, there is a move form a Th-1 to a Th-2 inflammatory profile [430] and not surprisingly, this effect has generated interest in the therapeutic potential of adenosine inhibitors in inflammatory diseases.

There remains some uncertainty over the role of genetic variation in ATIC and the subsequent influence on MTX effects as has been reviewed in chapter 1 and there are no reports on the functional effects of this polymorphism. Like the results here, the first report of ATIC 347C>G on MTX response in RA showed that the minor allele correlated with clinical response [423]. However a subsequent larger study demonstrated the exact opposite, namely the major allele was associated with superior clinical response [390]. These contentious findings have had some clarification from a recent GWAS examination that was applied to MTX therapy in RA. This showed that an intronic marker rs4673993 that was in complete LD with the ATIC 347C>G polymorphism also showed an association for clinical response with the minor allele, hence consistent with the first reported observation [431] and the results in this study. It is important to note that this GWAS study did not follow a hypothesis free approach; nevertheless it was the only positive finding. None of these studies have specifically reported on the influence of folate supplementation on these effects.

The results in this study draw attention to another animal model where the anti inflammatory effects of MTX were not abolished by adenosine receptor antagonists but instead by high doses of folic acid supplementation [319]. The doses of MTX used were considerably higher than in earlier experiments and is consistent with a landmark double blinded clinical trial [432]. These data provide an elegant illustration of the differences between low dose and high dose MTX therapy. There may be a folate dependent mechanistic influence of ATIC on MTX response that is currently undefined. The findings in this experiment show that the clinical effects of MTX therapy in carriers of the major ATIC 347C allele are higher in those individuals receiving oral folate supplementation. Not taking oral folates appear to reduce response rates in carriers of this allele. Faced with this novel effect, one may also hypothesize that the ATIC 347C>G polymorphism is in LD with another functional variant that influences MTX response in a folate status dependent way. Clearly, these findings suggest a need for further investigation of these effects.

4.5.4 Aldehyde Oxidase (AOX-1)

The expected effect of the polymorphism was not seen. The influence of Aldehyde oxidase on 7-OH MTX levels are seen in cancer chemotherapy and the lower dosages of MTX used in IBD may not be adequate. The proposed influence of the SLC19A1-1 variant on clinical efficacy was also not seen. Both hypotheses were discarded

4.5.5 Side effects

Using the pharmacogenetic model to study the correlation of genetic variation on side effect rates is a major rationale for the approach. However the process is fraught with difficulty as a number of the side effects are not attributable to single patho physiological mechanisms. Previous studies have tried to introduce a classification to side effects namely hematological, hepatic, gastro intestinal, skin and mucosa, neurological etc. While this is reasonable for data collection, analysis of the small numbers in each of these groups is not without difficulty. One is left having to merge some of these groups to attain meaningful numbers. This erodes the rationale of the exercise. In this thesis, the approach is of reporting all side effects and subsequently examining those that led to cessation of therapy. It was hoped that this approach will avoid some of the pitfalls of other approaches.

Individuals with the wild type ATIC 347G allele were more likely to be free of side effects if they were receiving folate supplementation. The *ATIC 347GG* genotype has been correlated with gastro intestinal intolerance in a study of patients treated with MTX for RA [389]. There was no stratification for folate status although 20% did not receive oral folates. In the data presented here, the numbers of affected individuals are small but there do not appear to be a particular predilection for a certain side effect in individuals with the three ATICC 347C>G genotypes.

4.6 Summary

In IBD, particularly UC, immunomodulators are often used after aminosalicylates have failed. When used, MTX traditionally follows AZA failure or intolerance. In recent practice, biologics such as infliximab may replace MTX as second line therapy. Importance should be attached to evidence in RA where the early use of MTX is nearly as effective as biologics [433]. Efficacy rates of 40 -50% may reflect the role MTX currently plays i.e., a second line drug used in potentially advanced or complicated disease. The role of pharmacogenetics is particularly important in such a situation as potentially greater benefit could be obtained if MTX could be offered as second line therapy in a group more likely to respond and infliximab be preferred second line therapy in the group more likely to fail therapy.

It is clear that MTX inhibits several key enzymes responsible for purine and pyrimidine synthesis. This affects DNA and RNA formation and is obviously cytotoxic. However, equally clearly, there is a difference between the effects of MTX as seen in cancer chemotherapy and those seen in the treatment of chronic inflammatory conditions such as RA, psoriasis and CD. Weekly low-dose MTX can suppress disease activity over a period of weeks and administration of folic acid every week does not affect clinical efficacy but reduces adverse drug events. There is no evidence that systemic toxicity in individuals receiving antifolates correlates to homocysteine levels, however folate supplementation abolishes homocysteinemia and also prevents side effects. These obvious discrepancies between what is expected and what was observed have been the subject of intense investigation.

Comprehensive reviews have provided some insight into this but there remains a need for further clarity in this area [312, 434]. It is possible that the effects of MTX therapy in IBD are unrelated to the folate pathway and may explain why these polymorphisms, in contrast to cancer chemotherapy, are not particularly influential in inflammatory diseases. This has now been the experience in a number of reports. Other mechanisms have been explored in RA and some evidence suggests that the effects of MTX *in vivo* may be due to T-cell apoptosis, modified cytokine production and reduced T cell proliferation. A role for intracellular reactive oxygen species (ROS) levels, inhibition of pyrimidine pathway enzymes and increasing CD 95 sensitivity of CD45+RO cells (activated T-cells) has been demonstrated and these may be significant [327]. Some of these alternative mechanisms are explored in subsequent chapters in this thesis.

5 The influence of the HLA-G 14 bp insertion / deletion polymorphism and the IL-10 promoter haplotype on response to MTX and AZA in individuals with IBD

5.1 Introduction

HLA-G is a non classical class I antigen and is biologically active. It creates a truncated form by alternate splicing that appears to mediate an anti inflammatory or a tolerogenic state. There is evidence that some of these effects are associated with elevated IL-10 levels and there may be a close correlation between the two. There is also evidence that the molecule is very relevant in the maintenance of a viable foetus, transplant graft survival and tumour cell viability. Genetic polymorphism in the 3' UTR, specifically a 14 base pair insertion destabilises the mRNA and may influence the effects of immunosuppressive medication [138].

5.2 Hypotheses

The hypotheses are:

- The HLA-G 14bp del/del polymorphism by means of increasing tolerance in intestinal mucosa in individuals with IBD predisposed them to response to immunomodulatory medication.
- High IL-10 producers (GCC haplotype) would be more likely to experience these anti inflammatory effects of the HLA-G 14bp ins/del polymorphism. There may in addition, be an independent effect of IL-10 on clinical response to immunosuppressive medication.

5.3 Aims

1. To examine the effects of he HLA-G 14bp ins/del polymorphism and the IL-10 haplotype on response to the study medication namely AZA and MTX in patients with IBD.
2. The HLA-G region is in LD with other areas in the chromosome 6, some of which influence susceptibility to IBD and influence joint disease in these patients. Hence the effect of the HLA-G 14 bp ins/del polymorphism on the susceptibility to UC and CD was examined.

5.4 Study Design

Three groups of patients were included in these experiments.

5.4.1 Methotrexate cohort

The patient cohort characterised and studied in the previous chapters on folate pathway enzyme pharmacogenetics is used here. (Table 5-1)

5.4.2 Azathioprine cohort

Patients for the AZA part of the study formed part of a prospective multicentre study of AZA for the treatment of IBD. It was designed to assess the impact of pre-treatment TPMT genotype and activity and subsequent red cell TGN levels on clinical outcome. The initial study reported on 215 individuals. All patients were aged between 16 and 80 years of age and had IBD diagnosed by standard criteria. After an initial TPMT assay to exclude low activity, patients were commenced on AZA 2mg/kg/day taken orally. The patients were followed up at weeks 4, 12 and 24 when they underwent an assessment of disease activity (Harvey Bradshaw Index for CD and Truelove and Witts for UC), side effects, TGN levels, full blood counts, liver function tests, ESR, CRP and a record of concomitant medication. Data was specifically collected on the use of steroids, 5-ASA. All those on steroids underwent a steroid taper over 12 weeks. Clinical response was defined as the achievement of each patient's stated treatment goal according to indication for treatment. Steroid withdrawal was defined as complete withdrawal of corticosteroids by 3 months and remaining off corticosteroids for a further 3 months. Maintenance of remission was defined as maintenance of normal inflammatory parameters and activity indices for 6 months. Remission of active disease was defined by normalization of disease activity indices at 6 months of treatment. Recourse to surgery, an alternative immunomodulator or a biologic agent was considered treatment failures. Adverse drug effects were recorded. The full protocol of this study has been reviewed and reported elsewhere [435].

A total of 124 patients completed the intended 6 months of therapy. The rest withdrew from therapy, mainly due to gastrointestinal intolerance. There was a significant positive correlation of

drug intolerance with TPMT intermediate activity. In those that completed therapy, there was also a negative correlation of clinical response with TPMT high activity and a positive correlation with therapeutic TGN levels. DNA in sufficient quantities for the experiments reported here were available in 97 patients of the 124 that completed 6 months of treatment. As this is a study of the markers of response to therapy, all those who were unable to complete a minimum of 6 months of treatment were excluded. They and the subjects whose DNA samples were exhausted form no part of this study.

The mean age was 36 years (range 18-80) and 43 were female. 48 (50%) patients were treated for CD of whom 11 (21 %) had ileal disease, 23 (44%) ileo-colonic and 14 (27%) colonic disease. Of the 49 patients with UC, 28 (55%) had pancolitis, and 21 (45%) had distal colitis. In the majority of patients (84/97, 85%), AZA was used as a steroid-sparing agent. Induction of remission of active disease was the indication in 3 (3%) patients, maintenance of remission in 10 (10%) patients (Table 5-2).

5.4.3 IBD susceptibility cohort

This cohort comprised 920 cases with CD, 654 UC and 665 controls. The cohort has previously been reported in a CD susceptibility study [436]. A multicentre research ethics committee approved the study of IBD susceptibility genotype in this cohort and the patients were recruited from Guy's and St. Thomas' Hospital, London, St. Mark's Hospital, London and the Royal Victoria Infirmary, Newcastle. All patients provided written informed consent. The diagnosis of IBD was established by standard clinical, radiological and endoscopic criteria. Phenotypic data was available on the majority of the individuals included (908 CD cases and 475 UC cases). This data is displayed in (Table 5-3). Matching of controls was not performed.
The population controls were obtained from the 1958 birth control cohort. The ethnicity of both cases and controls was 94% white Caucasian and 6% non Caucasian.

Table 5-1 Phenotype and therapeutic details of the patients included in the MTX pharmacogenetics experiments

Total Patients			201
Females			116 (57%)
Disease type	Crohn's Disease	All cases	145 (72%)
		Colonic Disease	40 (27%)
		Ileo Colonic	75 (52%)
		Ileal	20 (14%)
		Upper Intestinal	10 (6%)
		Perianal	41 (29%)
	Ulcerative Colitis	All cases	38 (19%)
		Left sided disease	18 (47%)
		Pancolitis	15 (39%)
		Proctitis	5 (13%)
	Indeterminate colitis	All cases	18 (9%)
Age at diagnosis	Crohn's disease		29 (Range 9 - 78 years)
	Ulcerative Colitis		38 (Range 19 - 75 years)
Extra Intestinal disease	Crohn's Disease	Arthritis	39 (26%)
		Erythema Nodosum	6 (4%)
		Ocular disease	10 (6%)
	Ulcerative Colitis	Arthritis	7 (18%)
		Erythema Nodosum	1 (2%)
Indication for MTX	All patients	Steroid sparing	111 (55%)
		Steroid resistance	15 (7%)
		Azathioprine failure	74 (36%)
		Patient preference	21 (10%)
Route of Administration	All patients	Oral	174 (86%)
		Sub cutaneous	3 (1.5%)
		IM induction / oral maintenance	22 (10%)
Mean Dosage	All patients		2.34 mg (7.5 to 25 mg/wk)
Folate supplementation		Yes	114
		No	67
		Data Unavailable	20
Response to Therapy	179 Patients	Yes	97 (54%)
		No	82 (46%)
Recourse to surgery	CD		76 (38%)
	UC		7 (3%)
	Indeterminate		1

Table 5-2 Phenotype and treatment characteristics of patients treated with azathioprine

Number of patients		97
Females		51 (52%)
Smoking		9/78 (12%)
Crohn's Disease		48 (50%)
	Ileal	11 (21%)
	Ileo Colonic	23 (44%)
	Fistula	5
Ulcerative Colitis		49 (50%)
	Pancolitis	28 (55%)
	Distal Colitis	21 (45%)
Indication for AZA	Steroid sparing	84
	Remission induction	3
	Remission maintenance	10
Response to AZA		57 (58%)
Concomitant medication	5-ASA	59 (60%)
	Steroids	74 (76%)
TPMT Genotype	Wild type	93
	Heterozygote	3
	Homozygote mutation	0
TPMT activity	0-10 pmol/hr/mg Hb	0
	<25	4
	25 - 50	70
TGN Levels (n=76)	>100 pmol/8×10$_8$ RBC	44
	<100 pmol/8×10$_8$ RBC	32

Table 5-3 The demographics and clinical characteristics of 908 patients with CD and 475 patients with UC

Characteristics		Number
CD	Sex : male/female	370 /535
	Median age at diagnosis (range)	25 (2-77)
	Disease behaviour	B1 = 383
		B2 = 326
		B3 = 186
UC	sex (male/female)	235/240
	Median age at diagnosis	30 (3-81)
	Disease extent	Proctitis = 85
		Left sided UC = 187
		Extensive UC = 203

5.4.4 Genotyping

The Forward Primer was RHG4 5′-GGAAGGAATGCAGTTCAGCATGA -3′ and the reverse Primer was GE14HLAG 5′-GTGATGGGCTGTTT AAAGTGTCACC -3′.
Thermocycling conditions were 92°C for 5 minutes, and 30 cycles at 92°C for 30 seconds, 64°C for 1 minute, and 72°C for 2 minutes, with a final elongation step of 72°C for 10 minutes. Reactions were set up in a 25µl mixture containing 100 ng of genomic DNA, 0.2 mmol/l NTPs, 1.5 mmol/l MgCl2, 10 pmol of each primer, and 1U of Taq polymerase.The amplified products were visualized by electrophoresis on a 2.5% agarose gel (Invitrogen, Paisley, Scotland, UK) containing ethidium bromide (0.5 µg/ml) and were run at 100v for 120 minutes.

5.4.5 Sequencing

The IL-10 promoter region containing the -1082 A>G (rs1800896), -819 C>T (rs1800871) and -592 C>A (rs1800872) polymorphisms was amplified using MolTaq Thermostable DNA polymerase 5 U/µl (Molzym GmBH&Co. KG, Bremen, Germany), in a total volume of 50 µl PCR reaction containing 0.5 µM of forward primer 5'-CACAAATCCAAGACAACACTACTAAGG-3', and reverse primer 5'-ATCCTCAAAGTTCCCAAGCAGC-3'. Primers were designed using the Primer3 v.0.4.0 website [393]. The thermocycler profile was 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72° for 1 min. Amplified PCR products were purified using QIAquick®PCR purification Kit (QIAGEN Ltd., Crawley, West Sussex, UK). Sequencing reactions were performed using the BigDye®terminator v3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK) with an additional two nested primers, forward 5'-GGGTGGAAGAAGTTGAAATAACAAGGAA-3', and reverse 5'-AAGTGCAGACTACTCTTACCCACTTC-3'. Sequencing reactions were cleaned using Agencourt®CleanSeq® (Beckman Coulter (UK) Ltd-Biomedical Research, High Wycombe, UK), and run on an ABI PRISM 3130x1 Genetic analyser (Applied Biosystems, Warrington, UK). Sequences were analysed by Mutation Surveyor Local v3.20 (Softgenetics LLC, State College, PA 16803 USA).

5.4.6 Statistical analysis

Statistical analysis was performed as described earlier.

5.5 Results

5.5.1 Methotrexate cohort

5.5.1.1 HLA-G 14 bp insertion/deletion polymorphism

All 201 patients were successfully genotyped. The genotype frequencies are comparable to control data in other independent studies [158,159]. Genotype frequencies were in Hardy-Weinberg equilibrium ($\chi 2 = 1.79$) (Table 5-4).

The insertion allele occurred significantly more commonly in patients who failed treatment compared to those who responded to treatment (80% vs. 54% respectively, p=0.0001, OR = 3.580, 95% CI: 1.5 to 8) (Table 5-5).

The variant HLA-G 14bp insertion allele had a frequency of 0.36 in those that responded to MTX therapy and 0.53 in those that did not (p=0.0007, OR= 0.48, CI 0.31 – 0.74) (Table 5-7). Folate supplementation did not influence this effect. There were no significant differences in the distribution of the IL10 -1082G allele between responders and non responders to therapy. Folate status did not have an effect. The type of IBD (UC vs CD had no influence on this effect (p=0.56). The insertion allele did not identify those who had a higher likelihood of failing AZA therapy in the past (p=0.88). UC and CD patients did not differ in genotype distribution or clinical response rates.

5.5.1.2 IL-10 -1082 A>G polymorphism

The mutation was present in 71 % of clinical responders and in 73% of non responders to MTX therapy. (p=0.86, OR = 1.06, CI= 0.5 – 2.05). Folate supplementation did not play a role in influencing clinical response rates (Table 5-5).

5.5.1.3 Interaction between the HLA-G 14bp genotype and the IL-10 1082A>G genotypes

A conditional analysis was performed to assess the influence these two polymorphisms may have on each other and on the primary outcome of clinical response. The results show a significant result for the haplotype of HLA-G14bp ins/del and IL10 -1082A>G (p=0.0036), but this is primarily from the HLA-G14bp ins/del contribution, as can be seen from the OR of 0.61 and 0.36 for the IL-10 1082G allele at the first marker (HLAG 14 bp ins). There is some indication that the OR for (IL-10 1082G – IL-10 1082G) is lower than for (IL-10 1082G – HLAG 14bp ins), which would indicate a role for IL-10 1082A>G in moderating the risk of HLA-G14bp ins/del. However, an analysis of IL-10 1082A>G conditional on HLA-G14bp ins/del) give no increase in fit (p-value = 0.34286) Fitting an additive model for risks across the two loci, (which reduces the degrees of freedom) does not help (p-value = 0.228543 for IL-10 1082A>G conditional on HLA-G14bp ins/del). (Table 5-6)

5.5.1.4 Side effects

Neither the HLAG 14bp insertion allele nor the IL10 -1082G allele influenced side effect rates. Folate supplementation also had no influence on this effect (Table 5-8).

5.5.2 Azathioprine cohort

5.5.2.1 The HLA-G 14 bp insertion/deletion polymorphism

All 97 patients were successfully genotyped and data are given in (Table 5-9)

The observed frequency of the deletion allele in the cohort was 54%. This is comparable to genotype frequencies in 665 population controls (55.3%) and control data in other independent studies [158,159]. Genotype frequencies were in Hardy-Weinberg equilibrium ($\chi^2 = 0.4201$). The homozygous del/del genotype frequency was significantly lower amongst patients who failed treatment compared to those who responded to treatment (12.5% vs. 44% respectively, p=0.001, OR = 5.469, 95% CI: 1.869 to 15.9).

The same pattern was also seen when the data was stratified by type of IBD. In those receiving AZA for CD, 2 of 19 (10%) non-responders were del/del homozygotes compared to 15 of 29 (52%) responders (OR = 9.107, 95% CI: 1.773 to 46). Similarly, 3 of 21 (14%) UC patients

failing to respond to treatment were homozygous for the del/del genotype compared to 10 of 27 (37%) of those who responded. UC and CD patients did not differ in genotype distribution (p=0.3851) or rate of clinical response (p=0.6). No differences were noted in age, sex, steroid use, 5-ASA use, pre-treatment TPMT, AZA dose and 24 week 6-TGN levels between the HLA-G 14bp del/del and insertion groups (Table 5-9).

5.5.2.2 IL-10 promoter haplotype

Genotyping was successful in all patients. All genotypes were in Hardy-Weinberg equilibrium. There were no significant differences in genotype distribution between clinical responders and patients who failed treatment or when divided into UC and CD subgroups. Although more patients with UC than CD had the -1082 G/G genotype [11/48 (23%) vs. 8/48 (17%)], this difference was not significant.

However, interestingly, no patients with the combined favorable *HLA-G 14bp del/del* genotype and the *IL-10 GCC/GCC* haplotype failed treatment. Possession of the favorable *HLA-G 14bp del/del* genotype and the IL-10 GCC haplotype conferred a significant likelihood of clinical response [18/20 (82%)] compared to those who lacked this combination [18/20 (82%) versus 39/76 (51%) respectively, p=0.001, OR = 8.4, 95% CI: 1.8 to 39) (Table 5-10).

5.5.2.3 The influence of TPMT activity and TGN levels on clinical response to azathioprine

As has been described previously, the HLA-G 14bp genotypes did not differ in the distribution of TPMT genotypes and the TGN levels at 24 weeks. It is recognised that TPMT levels correlate with clinical response and that TGN levels may do the same. As the findings of the study that forms the source of the patients in this experiment has found similar results, it is important to asses the influence of these variables on clinical response in this cohort. Exhaustion of DNA stocks led to the exclusion of 27 subjects who were suitable for inclusion (had completed 6 months of treatment).

TGN levels at 24 weeks were available in 76 individuals. A level of greater than 100pmol/ 8×10^8 RBCs was taken as predictive of clinical response in the original publication. Hence that has been used here. 44 patients achieved levels greater than this cut off. The TGN levels did not

correlate with clinical response in this group (p=0.23, OR= 1.891, CI = 0.7 -4.8). The high TGN patients were not different in distribution between the HLAG deletion individuals (High TGN levels in Response vs. treatment failure among HLA-G 14bp del carriers), p= 0.16, OR= 2.3, CI = 0.7 – 7.3).

TPMT levels correlated with therapeutic response in the study population. A TPMT level of more than 35pmol/hr/mg of haemoglobin was more frequent among clinical non- responder than in responders to AZA (p=0.02, OR =2.7, CI = 1.18 – 6.2). The number of patients with high TPMT levels were more among those with a HLAG 14bp insertion than in those without the insertion, but this difference fails to achieve significance (p=0.07, OR = 2.3, CI = 0.9 – 6).

5.6 IBD susceptibility cohort

All the genotypes were in HWE. The 14 bp del allele was not distributed significantly differently among the individuals with UC (p= 0.97, OR=1, CI= 1- 1.7) or CD (P= 0.9, OR = 0.99, CI = 1 – 1.4) when compared with the 1958 birth cohort controls. (Table 5-11, Table 5-12)

Table 5-4 SNP frequency information for HLA-G 14 bp ins/del and IL-10 -1082A>G in the MTX group

From dBSNP.http://www.ncbi.nlm.nih.gov/projects/SNP/.

The frequencies reported are of the homozygous mutation.

SNP	rs no	Freqency in Caucasians	Frequency in this study
IL-10-1082 A>G	1800896	0.283	0.24
HLA-G 14bp INS/DEL	1704	0.38	0.31

Table 5-5 The dominant influence of the HLA-G 14bp insertion/deletion polymorphism and the IL-10 1082A>G polymorphisms on clinical response.

The reported OR and p values are for the wild type genotype vs. the mutant heterozygotes and homozygotes

SNP	Response to MTX	No response	OR	CI	p
IL-10-1082 A>G	65/91 (71%)	64/88 (73%)	0.9	0.4 - 1.8	0.86
HLA-G 14bp INS/DEL	49/91 (54%)	71/88 (80%)	3.5	1.5 - 8	0.0001

Table 5-6 The influence of the haplotype of the HLAG 14bp insertion allele and the IL-10 1082 G allele on clinical response rates to MTX

CR is clinical responders and NR is non responders to therapy. OR and p values displayed are for the haplotype shown

Haplotype	Freq in CR	Freq in NR	OR	CI	Chi sq	P-value
HLAG14 bp ins - HLAG 14 bp ins	0.3235	0.2289	1	1	6.034	0.01403
HLAG14 bp ins - *IL10* -1082G	0.3139	0.2314	0.9597	0.4891 - 1.883	2.519	0.1125
IL10 -1082G - HLAG14 bp ins	0.215	0.2484	0.6122	0.3035 - 1.2	1.321	0.2505
IL10 - 1082G - *IL10* -1082 G	0.1477	0.2914	0.3586	0.1916 - 0.67	10.57	0.001149

Table 5-7 The allele frequencies of the examined genotypes and their distribution between clinical responders and non responders

Table A includes all the subjects, Table B are those that did not receive oral folate supplementation and Table C are those that did. In each case, the minor allele is the allele for which the frequency, estimated Odds Ratio and p value refers. All patients n=179, Folate supplemented n=103, Folate not supplemented n=58, folate status unknown n=18.

TABLE A

Gene	All patients				
	Response	Non Response	OR	CI	Pvalue
HLAG	0.3626	0.5398	0.4851	0.3177-0.7408	0.000733
IL10	0.4615	0.5227	0.7826	0.5167-1.185	0.246825

TABLE B

Gene	No folate supplements				
	Response	Non response	OR	CI	Pvalue
HLAG	0.3788	0.46	0.7158	0.3394 - 1.509	0.379451
IL10	0.5	0.48	1.083	0.5193 - 2.26	0.83102

TABLE C

Gene	Folate supplemented				
	Response	Non response	OR	CI	Pvalue
HLAG	0.3596	0.5326	0.4929	0.2814 - 0.8632	0.012735
IL10	0.4386	0.5652	0.601	0.3454 - 1.046	0.07039

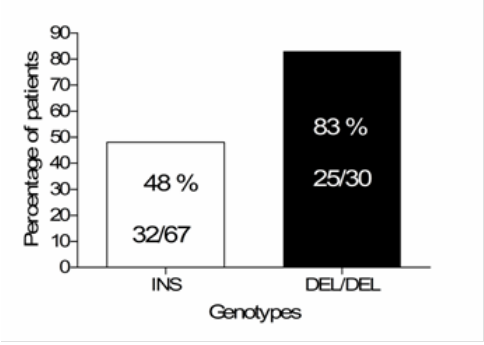


Figure 5-1 The effect of the HLA-G 14bp del/del genotype on the percentage of patients with IBD responding to treatment with AZA.

Individuals with the insertion allele had a 48% chance of response to medication as opposed to the del/del genotypes that had an 83% chance of success.

Table 5-8 The influence of the HLAG 14bp insertion allele and the *IL10*-1082G allele on side effect rates.

Table A is all patients, Table B is without folate and Table C is folate supplemented groups.

TABLE A

Gene	All Cases				
	Side-effects	No side effects	OR	CI	Pvalue
HLAG	0.5306	0.4375	1.453	0.9204 - 2.295	0.108217
IL10	0.8488	0.8618	0.9002	0.4588 - 1.766	0.761271

TABLE B

Gene	No folate supplementation				
	Side-effects	No side effects	OR	CI	Pvalue
HLAG	0.5667	0.4216	1.794	0.7886 - 4.083	0.161224
IL10	0.7857	0.8725	0.5356	0.183 - 1.568	0.267484

TABLE C

Gene	Folate supplementation				
	Side-effects	No side effects	OR	CI	Pvalue
HLAG	0.4815	0.4195	1.285	0.6961 - 2.371	0.423475
IL10	0.8913	0.8563	1.376	0.4959 - 3.817	0.529185

Table 5-9 Genotype frequencies of HLAG 14 bp Ins/del and the IL-10 promoter polymorphisms stratified for disease type and clinical response to azathioprine.

Genotype		IBD		UC		CD	
		Response	***Failure***	***Response***	***Failure***	***Response***	***Failure***
HLA-G 14Bp ins/del	ins/ins	7	15	3	8	4	7
	ins/del	25	20	14	10	11	10
	del/del	25	5	10	3	15	2
	p(del/del vs. ins)	p=0.0015		p=0.11		p=0.005	
***IL-10* -1082 A>G**	A/A	15	10	7	7	12	6
	A/G	31	22	14	9	13	10
	G/G	11	8	6	4	5	4
	p=(G/G vs A)	p=1.00		p= 1.00		p=1.00	
***IL-10* -819 C>T**	C/C	28	24	14	16	14	8
	C/T	24	14	12	5	12	9
	T/T	5	2	1	0	4	2
	p=(TT vs. C)	p=0.6		p=1.00		p=1.00	
***IL-10* - 592 C>A**	C/C	28	24	14	16	14	8
	A/C	24	14	12	5	12	9
	A/A	5	2	1	0	4	2
	p= (AA vs. C)	p=0.6		p=1.00		p=1.00	

Table 5-10 The influence of the high producer HLAG1bp and IL10 promoter haplotypes on clinical response to AZA

HLA-G 14bp ins/del (HLA-G 14 base pair insertion/deletion polymorphism), IBD (Inflammatory bowel disease), UC (Ulcerative colitis), CD (Crohn's disease). The high producer promoter haplotype is GCC. The table compares the low producer ATA to the GCC both as heterozygotes and homozygotes.

	ATA		GCC		GCC/GCC	
	Response	Failure	Response	Failure	Response	Failure
All patients	15	8	38	27	11	8
14bp Ins	9	6	20	25	5	8
14bp Del/del	6	2	18	2	6	0

Table 5-11 The distribution of HLAG 14 bp genotypes in the IBD cohort and normal controls

MARKER	Patient Group	Number of individuals			
		Ins/del	ins/ins	del/del	χ2 (HWE)
HLAG 14bp	IBD	337	732	506	0.019
	Controls	146	303	216	0.0418
	CD	205	409	306	0.0026
	UC	132	322	200	0.937

Table 5-12 Differences in the prevalence of the HLAG 14 bp ins/del polymorphism between IBD patients and controls from the 1958 Birth Cohort

	ALLELE	CONTROLS	CASES	TOTAL	p	OR	(95% CI)
CD	DEL	735	1021	1756	0.9	0.99	(0.86-1.14)
	INS	595	819	1414			
UC	DEL	735	722	1457	0.97	1	(0.86-1.17)
	INS	595	586	1181			

5.7 Discussion:

Previous studies in RA have shown that the HLA-G 14bp deletion polymorphism is associated with MTX response (p= 0.009; OR= 2.46, 95% CI 1.26 to 4.84) and the expression of sHLA-G in LPS stimulated PBMCs cultured with MTX *in vitro* is highest in subjects with the homozygous 14bp deletion [437]. There is evidence that the HLA-G 14bp ins/del polymorphism influences the response to post-organ transplant immune suppression and that sHLA-G levels are up regulated by this treatment [438, 439].

The MTX group have demonstrated a powerful influence of the studied polymorphism that is apparently folate independent. It is interesting that the genotype was not under represented in the MTX group as the majority of patients had arrived at MTX after AZA therapy. However, the bulk of these were intolerant to AZA rather than unresponsive to it and that may explain this finding. IL-10 promoter polymorphisms do not modify the influence and the dose of MTX used has not modified the effect.

The study presented in this chapter has demonstrated an association with response to AZA and MTX treatment in patients with IBD. Presence of the HLA-G 14bp insertion polymorphism is associated with a significantly reduced likelihood of successful response to AZA treatment irrespective of disease type, but the influence appears stronger in CD compared to UC. No such differences were seen in the MTX treated group but UC patients were not very many.

The immunosuppressive effects of AZA may be due the integration of 6-TGN's into DNA leading to DNA fragility, failure of repair mechanisms and inhibition of replication [440]. These effects are almost certainly less relevant at the lower dosages of the drug used in the treatment of inflammatory conditions such as IBD [441]. In this setting, more recent evidence suggests that the drug may act via 6-Thio-GTP and its inhibitory effects on Rac1 and subsequently on T cells leading to apoptosis [255, 442]. The 24 week TGN levels did not correlate with clinical response. Whilst as described earlier, the mechanism of action of thiopurine drugs is poorly understood, there is no doubt that the generation of high levels of 6-TGN predisposes to haematological toxicity [443] and that high levels of MeMP can predispose to hepatoxicity [273, 444]. 6-TGN levels above 450 pmol/8×10^{8} are associated with leucopenia in TPMT deficient patients started on standard dosage therapy [445, 446]. However on dose escalation, WBC counts trends are relatively modest (8.6 down to 6.9 $\times10^{3)}$ and patients experience significant benefits in reducing

disease activity indices and steroid requirements at TGN levels that do not cause leucopenia [447]. However, the application of TGN levels in targeted dose adjustment of AZA has been difficult to justify in light of significantly contradictory trials [212]. In light of the mechanism of the action of AZA that could include TGN mediated modulation of Rac-1, this is somewhat difficult to reconcile and may be the effect of small sample sizes and variable recruitment criteria. Furthermore, the technical aspects of TGN measurement are difficult and are not consistent across different laboratories [264]. A meta analysis has addressed some of these difficulties and demonstrated that TGN levels more than 230 pmol/8×10^8 are more commonly found in individuals responding to AZA/6-MP (p= 0.006 for clinical response , 95%CI) [448]. This is not the experience with the limited data available in this group,

At first glance the TPMT levels appear to contribute significantly to clinical response in this study and may be a confounding influence. An important consideration is that the TPMT heterozygotes were in effect excluded form this study group due to the design of therapeutic intervention. A large number of heterozygotic individuals were intolerant of the fixed full dosing used and withdrew before the conclusion of the trial. Also, although the p values denote significance, the confidence intervals are wide. These limitations make further analysis of this observation difficult. Importantly, the TPMT levels or genotypes were not distributed differently between the HLA-G 14bp genotypes and conditional analysis suggests that the major contribution to clinical response is from the HLA-G genotype.

Intestinal epithelial cells (IECs) are important in innate and adaptative immunity and HLA-G may protect IECs from NK-mediated cell lysis [449]. PBMCs that express HLA-G, influence decidual cells *in vitro* leading to a shift from Th1 to Th2 cytokine expression (reduced TNF-α and IFN-γ and increased IL- 4 [450]. IBD has traditionally been considered a consequence of an imbalance in the interaction between Th1 (via secretion of IFN-γ, TNF-α, IL-2) and Th2 (via IL-10 and IL-4) mechanisms [451]. An HLA-G mediated shift from Th1 to Th2 expression and the implied resolution of an acute inflammatory state may explain the correlation with response to anti-inflammatory or immuno-modulatory therapy. It has been postulated that HLA-G, through the expression of IL-10, or sHLA-G, mediates a protective effect at the mucosal interface in the gut [449]. Enhancement of this effect may therefore be another potential mechanism of action of AZA and MTX.

IL-10 production in PBMC cultures has previously been noted to be highest in individuals with the HLA-G 14bp ins/ins genotype [180] and serum IL-10 levels have been implicated in modifying response to immunomodulators in RA [452]. HLA-G and IL-10 appear to be closely interrelated in their immunological influence [180]. Hence, it is possible that the HLA-G 14bp insertion destabilises mRNA and impairs sHLA-G production. When this is associated with impaired IL-10 secretion, the response to immunomodulatory drug therapy may be diminished. Although limited by small numbers in sub-group analysis, the data in this study appears to support this hypothesis. The combination of the favourable HLA-G 14 bp deletion and the IL-10 GCC promoter haplotype favoured a clinical response to AZA therapy in IBD patients.

Interestingly, this study revealed that the influence of HLA-G 14bp ins/del polymorphism on response to AZA medication was less significant for UC than for CD. LPS-stimulated PBMCs from patients with UC express more sHLA-G in association with lower IL-10 production than those from individuals with CD [159]. No correlations with genotype were made. Immunohistochemical studies of gut mucosa have shown that all patients with UC express HLA-G and IL-10 at the apical surface of intestinal epithelial cells (IEC) and the crypts of Lieberkuhn in both non-inflamed and inflamed areas. This was not seen in patients with CD [451]. Hence, there is clearly a difference in the expression of HLA-G both *in vivo* and *in vitro* however mechanistic inferences are difficult to make currently.

In light of extensive LD across the HLA region and the known association of this area with susceptibility to colonic CD and joint disease in IBD patients, it is reassuring that the 14 bp ins/del polymorphism is not increased in frequency amongst those with CD, UC or IBD (UC +CD) when compared with controls. This is particularly relevant because of the previously reported association of the 14 bp ins/ins genotype with ileo-caecal resection in individuals with CD [177].

In conclusion, the 14bp insertion/deletion polymorphism in HLA-G influences individual response to AZA and MTX in IBD. The polymorphism does not influence susceptibility to UC or CD and does not correlate with disease location in CD. Possession of the insertion allele significantly reduces the likelihood of successful clinical response. The hypothesis that there is a further independent correlation between AZA response and IL-10 promoter haplotype is not supported by our data but IL-10 and HLA-G genotypes may act together to influence response.

Further investigation of these immunomodulatory effects is likely to improve our understanding of the influence of these molecules in IBD and other chronic inflammatory diseases.

6 IL-10 and soluble HLA-G expression in vitro is influenced by genotype/haplotype and is different between samples co-incubated with 6-mercaptopurine and methotrexate

6.1 Introduction

Like other HLA molecules, soluble HLA-G (sHLA-G) is detectable in body fluids [148] and decreased sHLA-G correlates with adverse foetal outcomes [453]. Conversely, increased sHLA-G levels are associated with an improved pregnancy outcome and improved graft acceptance after cardiac and liver-kidney transplantation [150, 151]. Expression of sHLA-G in transplant recipients is induced by therapy with steroids, tacrolimus and ciclosporin and correlates with improved graft survival [439]. *In vitro* experiments on peripheral blood mononuclear cells (PBMCs) have shown a similar effect of MTX in RA [437]. The reduced sHLA-G expression reported in patients with UC, as compared to healthy subjects and those with CD was related to a reduction in IL-10 secretion [159]. Hence although, an influence of the interaction between the genotypes investigated in the previous chapter could not be found on clinical response to immunomodulatory medication, HLA-G and IL-10 may be closely interrelated [180].

6.2 Hypotheses

The hypotheses examined in this chapter are

1. The expression of sHLA-G would be diminished in individuals possessing the 14 bp insertion.
2. The expression of IL-10 would be influenced by the promoter haplotypes examined and the high producer GCC would express the highest levels.
3. The expression of IL-10 would be higher in individuals with the HLA-G 14bp del/del genotype than in those with the other genotypes.
4. The expression of sHLA-G and IL-10 from immune cells would be influenced by exposure to MTX and 6-MP and differences would be seen between the high producer and low producer HLA-G and IL-10 genotypes or haplotypes. A dose effect would be evident.

5. There would be an increase in IL-10 and a decrease in pro inflammatory cytokines such as TNF-α and IL-1β among those individuals who expressed the highest sHLA-G levels when compared with those who expressed the lowest levels.

6.3 Aims

1. To asses the level of IL-10 expression in LPS stimulated PBMCs at 24 Hours and 48 hours after incubation with varying concentrations of AZA and 6-MP.
2. To assess the level of sHLA-G expression in LPS stimulated PBMC cultures incubated with varying concentrations with AZA and 6-MP.
3. To assess the differences in the levels of TNF-α, IL-23, IL-6, IL-1β, IL-18 and IL-8 in the above cultures and compare the differences in these levels between high sHLA-G and low sHLA-G cultures.

6.4 Study design and methods

Seventeen Caucasian, female volunteers, aged between 25 and 55 years, were recruited for PBMC experiments. IL-10 haplotyping failed in one individual. This sample has been used for analysis involving the HLA-G14bp ins/del genotypes which was successful. Hence IL-10 analysis is performed on 16 individuals and sHLA-G on 17 individuals. None of the volunteers had co existing medical conditions or were on any medications. Written informed consent was obtained from all participants. Ethical approval for the additional pharmacogenetic work and cell culture experiments was granted by Bexley and Greenwich LREC (06/Q0707/84).

The monocytes were isolated and expression of sHLA-G and IL-10 was compared between cultures with and without MTX alone or 6-MP alone after LPS challenge and incubated for 48 h. MTX and 6-MP in concentrations of 0.25, 0.5 and 1 μM were studied. HLA-G 14bp ins/del genotyping was performed as described (page no: 96). Sequencing of the IL-10 promoter region containing the -1082 A>G (rs1800896), -819 C>T (rs1800871) and -592 C>A (rs1800872) polymorphisms was performed. For the assay of other cytokines, of the samples already collected, the 3 individuals with the highest baseline sHLA-G levels were selected from amongst the high producer genotypes and those with the lowest sHLA-G levels from the low producer genotypes. The low producer genotypes were those with the 14bp insertion allele. Samples from these six

individuals were used for this experiment. The samples analysed were as described before. Only samples incubated for 48 hours were used. The samples were analyzed by Flowcytomix assay. Levels of IL-23, TNF-α, IL-8, IL-18 and IL-1β were analysed.

Statistical analysis

The values for the different samples that were analysed in duplicate were used and means were calculated. The mean values were used for all calculations. The non-parametric Mann-Whitney U test was used to test for an effect by genotype on the median change in sHLA-G and IL-10 values after cell culture with 6-MP. The two-way ANOVA for repeated measures was used to test for significance in differences in IL-10 expression between samples incubated with and without 6-MP and MTX. A p value less than 0.05 was taken to indicate statistical significance for all analysis.

The t Test with Welch's correction was used for assessment of the significance of the effect of genotype on the mean differences in sHLA-G, IL-10, TNF-α, IL-23, IL8, IL-6 IL-18 and IL-1β expression.

6.5 Results

All cell cultures were viable at all drug concentrations used. This was assessed by trypan blue staining.

6.5.1 Interleukin-10 (IL-10)

As described earlier, IL-10 haplotyping failed in 1 of the 17 individuals recruited. Hence data is presented for 16 individuals. No significant IL-10 expression was seen in un-stimulated samples. LPS stimulation increased IL-10 levels and levels at 24 hours were significantly higher than un-stimulated samples. ($p=<0.0001$, Mann Whitney U for un-stimulated v/s 24 hour samples). (Figure 6-1) However all the levels were increased and differences were difficult to elicit. Over the second 24 hours, the IL-10 levels were lower ($p=0.0017$, MWU for means 24 hrs vs. 48 hrs). The IL-10 levels in 48 hour samples were also significantly greater than un-stimulated samples ($p=<0.0001$, MWU for unstimulated vs. 48 hour incubation) and hence 48 hour samples were used in all the analysis.

No significant differences were seen in the baseline and 48 hour levels of expression between the high producer and the low producer IL-10 promoter haplotypes after LPS stimulation. The IL-10 levels were 20% higher in the high producer haplotypes ($p=0.7$ MWU for means, IL10 GCC vs non GCC haplotype (Figure 6-2).

6.5.1.1 The effects of 6-mercaptopurine on IL-10 expression

No differences are seen between the IL-10 levels in the low producer and high producer haplotypes when 6-MP at 0.25 μM is used. This increases to 20% higher IL-10 levels in the high producer haplotypes when 0.5 μM 6-MP is used. A further increase is seen when 1 μM 6-MP is used and the levels are 50% higher in the high producer haplotypes. Hence, the levels over the second 24 hours of cell culture are apparently influenced by drug dosages. Lower expression levels are seen in the 6-MP dose of 0.25 μM when compared with the dose of 1 μM However, this finding does not reach significance ($p=0.7$, with the parametric t Test with Welch correction for variance for comparison of means). There is also an apparent influence of the promoter

haplotypes. It is possible that small sample sizes have not allowed significance to be evident (p=0.2, unpaired two tailed T test for comparison of the difference in means of IL-10 expression between the 6 MP doses 0.25Mm and 1 μM in GCC and non GCC) , nevertheless a trend is hinted at in this graph (Figure 6-4). This experiment may indicate that IL-10 levels are influenced by 6-MP in PBMCs more so in individuals with the high producer GCC haplotype of the promoter region.

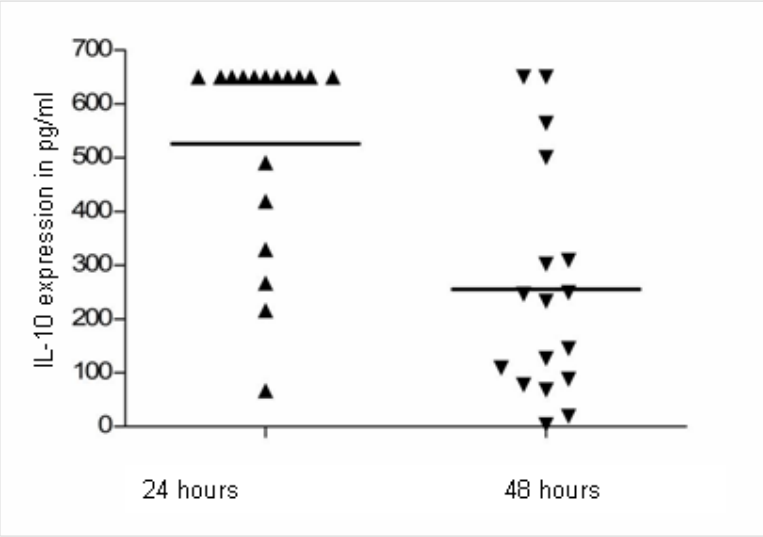
6.5.1.2 Methotrexate

The results vary from those seen with 6-MP and similarly, significance is not achieved. After 48 hours of culture, the high producer haplotypes express more IL-10 but a dose effect is not as evident as in 6-MP with increasing drug concentrations. The low producer haplotypes exposed to the study medication *in vitro* over 48 hours express 40% lower IL-10 levels than the high producer haplotypes with 0.25 μM MTX, 52% lower with 0.5 μM MTX and 1 μM MTX (Figure 6-5) (p= 0.4, Unpaired t Test for High producer vs. low producers for difference in IL-10 between dose 0.25 μM MTX and 0.5 μM MTX) (Table 6-2).

Although, this effect did not reach statistical significance these experiments suggest that there may be a pharmacogenetic influence of the IL-10 haplotypes on the dose response seen in the use of 6-MP and MTX for inflammatory conditions. IL-10 expression levels appear different between PBMC cultures with 0.25 μM MTX and 1 μM MTX when stratified for IL-10 promoter Haplotype GCC vs. non GCC (p= not significant) (Figure 6-5).

6.5.1.3 The influence of the HLA-G 14 bp insertion/deletion genotype on IL-10 expression and differences between the two studied pharmacological agents.

This hypothesis was examined in both the study groups detailed above. These experiments have not demonstrated a significant influence of these genotypes on baseline IL-10 expression after LPS stimulation at 24 hours or 48 hours in neither the 6-MP group nor the MTX group. Also, no differences were seen with either drug in varying concentrations. (Table 6-3)



This graph shows that levels decline over 48 hours and significantly lower levels that those at 24 hours are seen (p=0.0017). Levels are in pg/ml.

Figure 6-1 IL-10 expression levels are significantly increased after LPS stimulation.

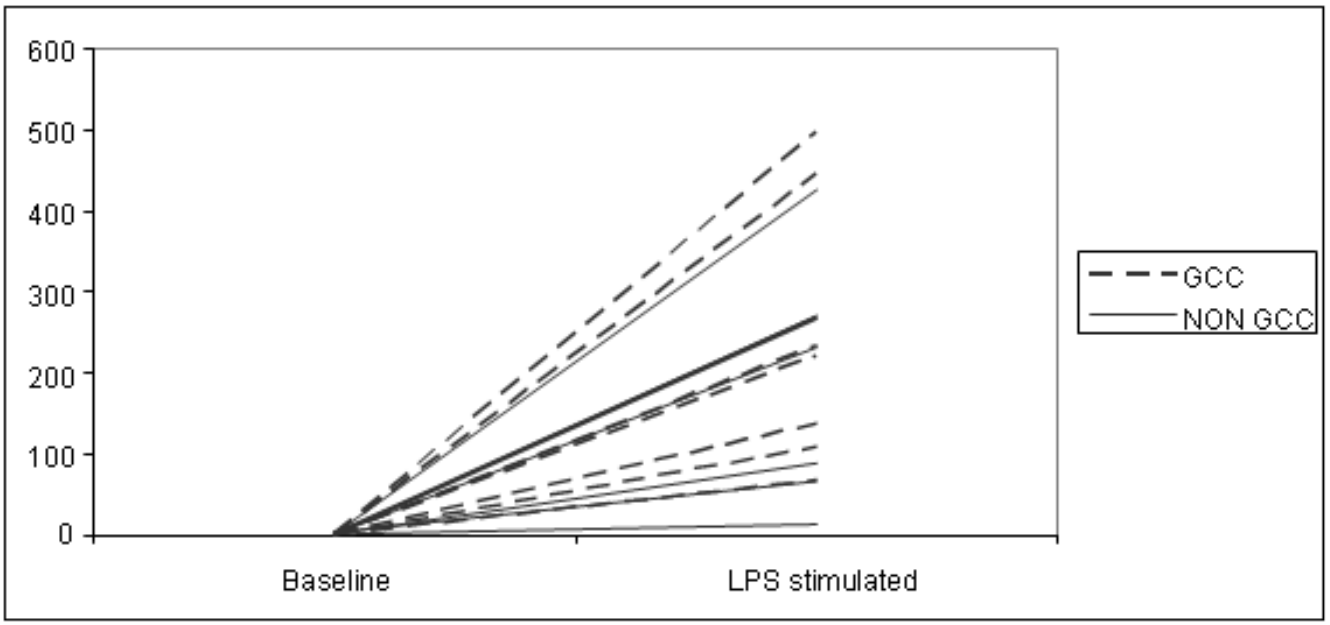
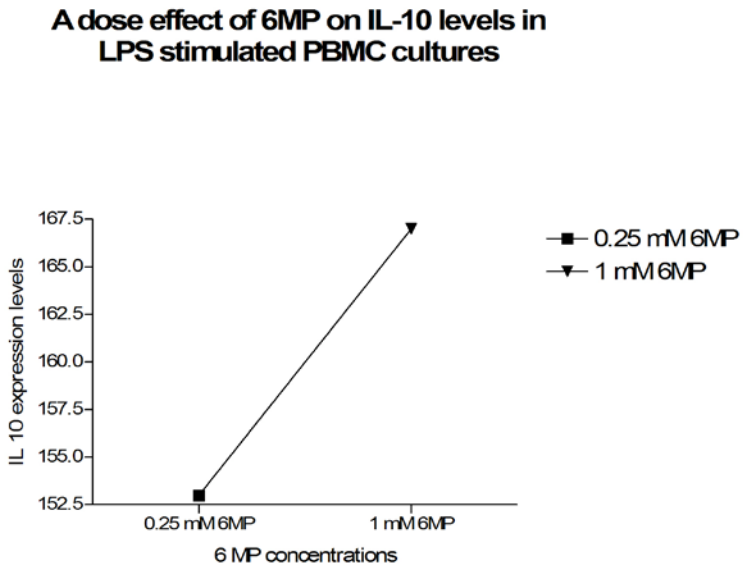
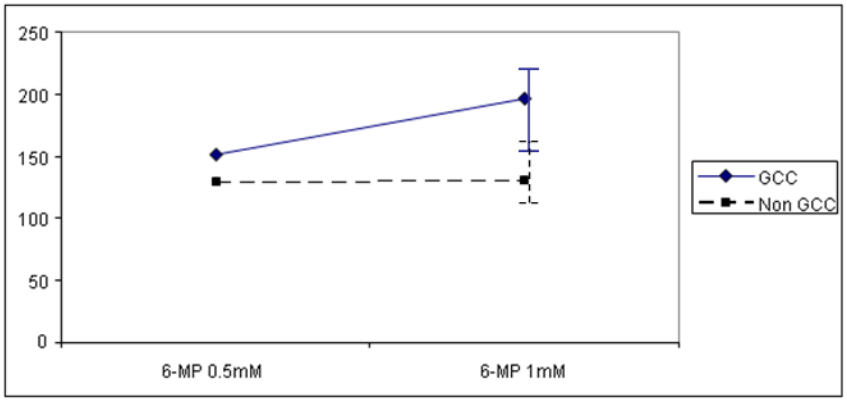


Figure 6-2 The IL-10 expression levels were not significantly different between GCC and non GCC haplotypes at 48 hours.



The cell cultures were stimulated with LPS and incubated without immunomodulatory medication. Levels are in pg/ml.

Figure 6-3 IL-10 levels expressed by PBMCs are apparently influenced by 6-MP after LPS stimulation. No drugs are used in this culture.



Lower doses of 6-MP are associated with lower levels of detectable IL-10 (p=not significant). All levels in pg/ml.

Figure 6-4 Expression of IL-10 are greater in individuals with the GCC promoter haplotype when exposed to 6-MP and are increased by greater drug concentrations

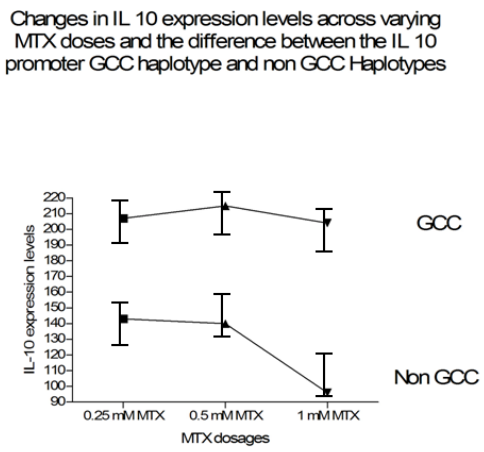


Figure 6-5 IL-10 levels after MTX incubation showing a dose effect in the high producer GCC haplotype

6.5.2 sHLA-G Expression

6.5.2.1 6-mercaptopurine co incubation

All cell cultures were viable at all drug concentrations used. No significant differences in sHLA-G expression were noted between genotypes at baseline (p=0.6) and after LPS stimulation (p=0.4). There were no significant differences between mean sHLA-G expression levels in the un-stimulated blank samples and those un-stimulated samples incubated with 6-MP. (Table 6-4)

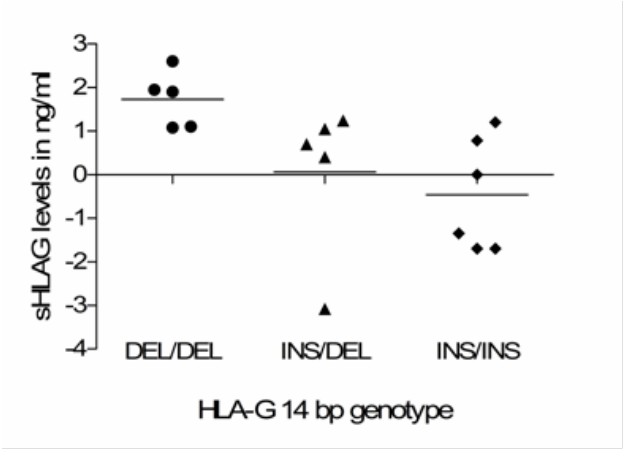
LPS stimulation increased sHLA-G levels in all genotypes. sHLA-G concentrations ranged from 1-6.5ng/ml (Median = 3.065, Mean = 3.242, SEM=0.4) in LPS stimulated cultures and between 0.2 -6.0ng/ml (Median = 3.8, Mean = 3.6, SEM = 0.39) after incubation with 1 μM 6-MP. Significant differences were noted in sHLA-G expression after 48 hours incubation with 1 μM 6-MP between carriers of the 14bp del/del genotype and the insertion allele. The greatest increases were seen in those with the del/del genotype (mean increase = 1.63ng/ml). The increase was less marked in the ins/del genotype (mean increase = 0.06ng/ml) and a decrease was noted in

the ins/ins genotype (mean decrease = 0.46ng/ml) (Figure 6-6). These differences were statistically significant (p=0.02 , unpaired t Test comparing mean difference in sHLA-G expression levels between LPS with and without 1Mm 6mp in del/del with the ins/ins genotype) (Table 6-4)

6.5.2.2 Methotrexate co-incubation

Here concentrations of 0.5 μM and 1 μM of MTX were studied. No significant differences in sHLA-G expression were noted between genotypes at baseline (p=0.6) and after LPS stimulation (p=0.4). LPS stimulation increased sHLA-G levels in all genotypes. sHLA-G concentrations ranged from 1-6.5ng/ml (Median = 3.065, Mean = 3.242, SEM=0.4) in LPS stimulated cultures and between 0.2 -6.0ng/ml (Median = 3.8, Mean = 3.6, SEM = 0.39) after incubation with 1 μM MTX. Significant differences were noted in sHLA-G expression after 48 hours incubation with 1 μM MTX between carriers of the 14bp del/del genotype and the insertion allele (Table 6-4). The greatest increases were seen in those with the del/del genotype (mean increase = 2.53ng/ml). The levels dropped in the ins/del genotypes (mean decrease = 0.54 ng/ml) and a further decrease was noted in the ins/ins genotype (mean decrease = 1.96 ng/ml). These differences were statistically significant (p=0.01, unpaired t Test comparing all three genotypes) (Figure 6-7).

Concentrations of 0.5 μM and 1 μM of MTX were studied and there appears to be a dose dependent increase in sHLA-G expression levels in the carriers of the del/del genotype that is not seen in the others. Indeed, in other individuals, the effect is one of a relative decline over the two dosages used after 48 ours of incubation (Figure 6-8).



Stimulated and co-incubated PBMCs from the high producer HLA-G 14bp del/del genotypes produced significantly more sHLA-G than the low producer ins/del genotype and the ins/ins genotype when exposed to 6-MP.

Figure 6-6 Comparison of the differences in mean increase in sHLA-G expression (ng/ml) between the 14bp del/del, ins/del and ins/ins genotypes after incubation of LPS stimulated PBMCs with 1 µM 6-MP

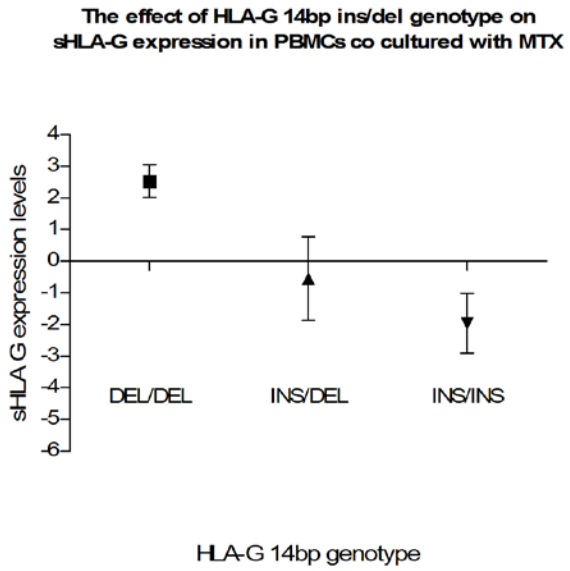


Figure 6-7 The effect of the HLA-G in/del genotype on the expression levels of sHLA –G in PBMCs cultured with 1 µM concentration of MTX

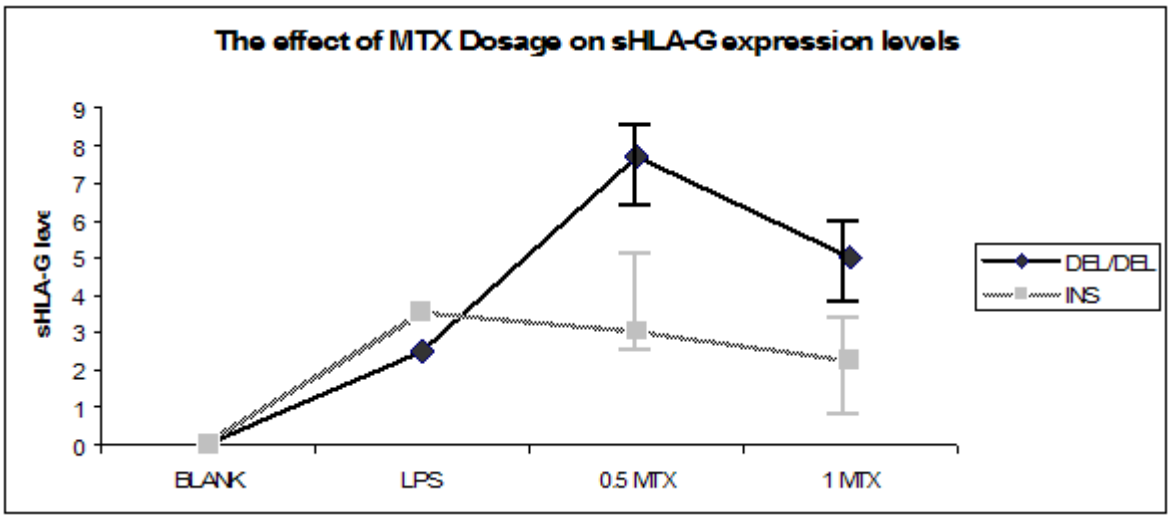


Figure 6-8 Demonstration of the effect of increasing MTX dosage on the expression levels of sHLA-G compared between carriers of the del/del genotype and the insertion allele

6.5.3 Other Cytokines

The samples were selected and analysed as described earlier. The data is displayed in tables Table 6-5 and Table 6-6. For statistical analysis, the data is displayed as the difference in cytokine expression between the un-stimulated and LPS stimulated samples. Subsequently the Mean and standard deviations of the cytokine levels in each of the sample groups for both the high s-HLA-G producer and low s-HLAG producer groups were calculated. The data for all samples is displayed, and analysis of statistical significance has been performed on samples co-incubated with 1 μM 6-MP or 1 μM MTX. The data for 6-MP and MTX is displayed in the appendices.

6-mercaptopurine group

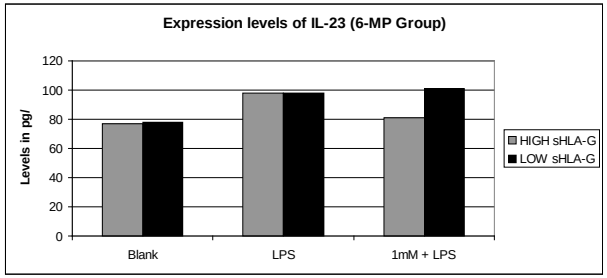
The data demonstrates some clear trends in the levels of cytokines. LPS stimulation increased all the levels of the examined cytokines irrespective of low or high sHLA-G status. When the samples were co incubated with 1 μM 6-MP, some differences were seen between the high and low sHLA-G groups. When LPS stimulated samples are exposed to 6-MP for 48 hours, IL-23 levels drop from the LPS stimulated baseline by 17% in the high sHLA-G group and are unchanged in the low sHLA-G group (p=0.2). The levels of TNF-α drop the low sHLA-G group by 14% and rise by 35% in the high sHLA-G group (p=0.39). No differences are seen between the low and high groups with IL-8 (p=0.2). IL-6 levels in the high sHLA-G group rise by 2% and drop in the low group by 7% (p=0.3). IL-18 levels in the high sHLA-G group rise by 15% and in the low group drop by 15% (p=0.5). IL-1β levels in the high sHLA-G group drop by 7% and in the low sHLA-G group rise by 5%. This data is demonstrated in Figure 6-9.

Methotrexate group.

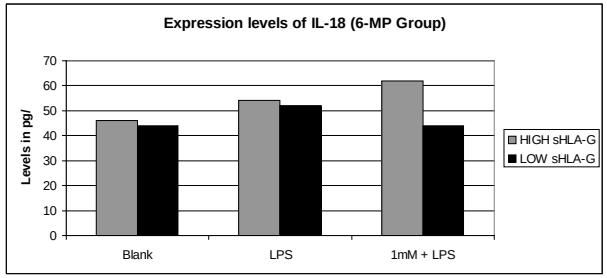
Here the data demonstrates some differences in trends in the levels of cytokines. LPS stimulation increased all the levels of the examined cytokines irrespective of low or high sHLA-G status. When the samples were co incubated with 1 μM MTX for 48 hours, IL-23 levels drop from the LPS stimulated baseline by 8% in the high sHLA-G group and by 10% in the low sHLA-G group (p=0.9). The levels of TNF-α drop in the high sHLA-G group by 15% and there is no change in the low sHLA-G group (p=0.6). No differences are seen between the low and high groups with IL-8 (p=0.4). IL-6 levels in the high sHLA-G group drop by 33% and are unchanged in the low group by (p=0.1). IL-18 levels in the high sHLA-G group drop by 17% and in the low group

drop it is unchanged (p=0.5). IL-1β levels in the high sHLA-G group drop by 35% and in the low sHLA-G group rise by 14% (p=0.2).

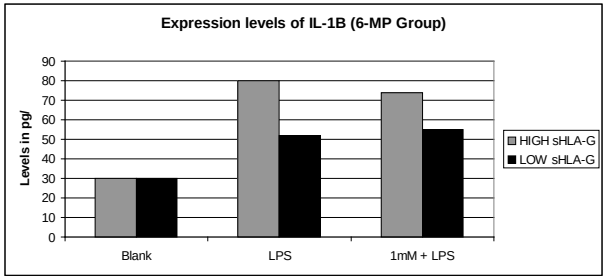
Hence, these experiments demonstrate that the levels of cytokines seen in LPS stimulated samples are not different at baseline between the low and high sHLA-G groups. On the other hand although significance is not achieved, the exposure of these samples to MTX and to a lesser extent 6-MP appear to influence the cytokine levels differently between these two groups. The numbers of individuals in this experiment is small and that may have influenced the significance levels seen. Nevertheless, in the MTX group the group of individuals who express the higher levels of sHLA-G and may be a group who respond well to immunosuppression if they had IBD, appear to have lower levels of TNF-α, IL-6, IL-18 and IL-1β. It is tempting to hypothesise that these changes predispose to a resolution of the inflammatory state. Such changes are not seen in the 6-MP group and indeed TNF-α and IL-18 levels are higher in the high sHLA-G group. The similarity of the effect of the 14 bp ins/del polymorphism on response to immunosuppression with 6-MP and MTX may not imply that the mechanism of these effects is also similar.



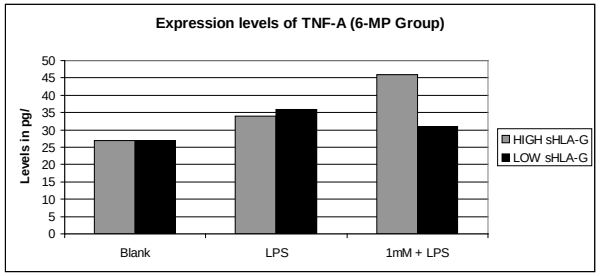
IL-23 Expression differences between the high sHLA-G and low sHLA-G group



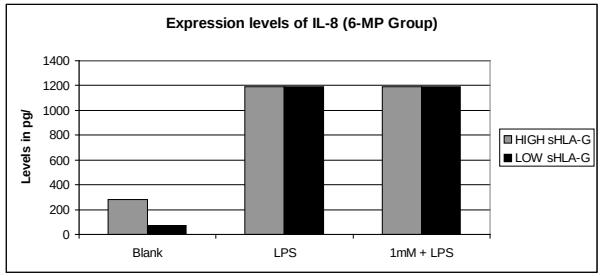
IL-18 Expression differences between the high sHLA-G and low sHLA-G group



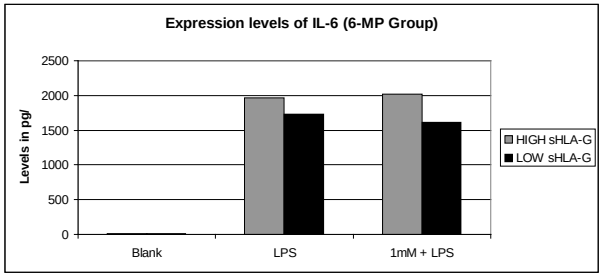
IL-1β Expression differences between the high sHLA-G and low sHLA-G group



TNF-α Expression differences between the high sHLA-G and low sHLA-G group



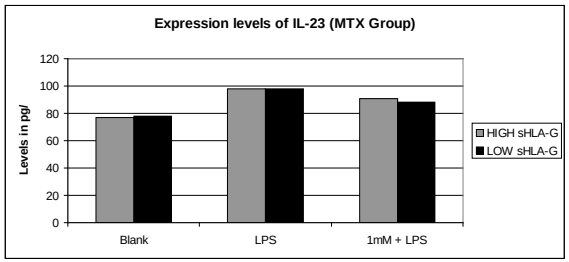
IL-8 Expression differences between the high sHLA-G and low sHLA-G group



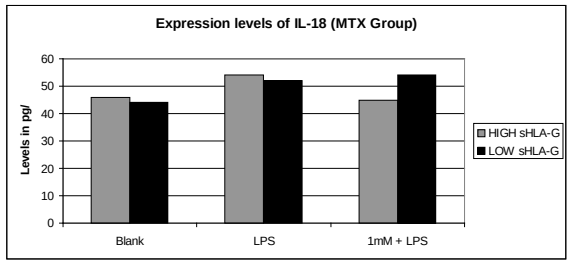
IL-6 Expression differences between the high sHLA-G and low sHLA-G group

Figure 6-9 Differences in the expression levels of the examined cytokines IL-23, IL-18, IL-1β, TNF-α, IL-6 and IL-8 after co incubation 1 µM 6-MP.

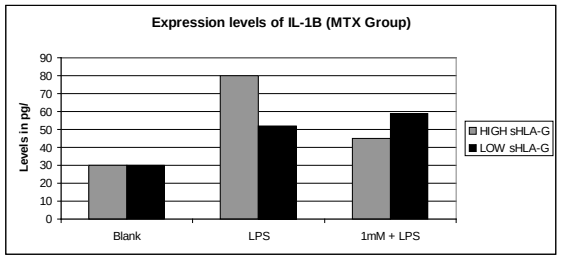
The low sHLA-G and high sHLA-G groups are being compared. 6-MP (6-mercaptopurine), TNF-alpha (Tumour necrosis factor-alpha), Interleukin 23 (IL-23), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interleukin 18 (IL-18), Interleukin 1 -beta (IL-1B)



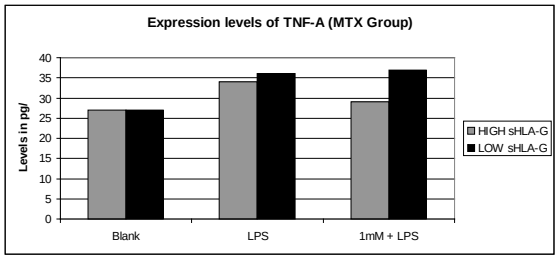
IL-23 Expression differences between the high sHLA-G and low sHLA-G group



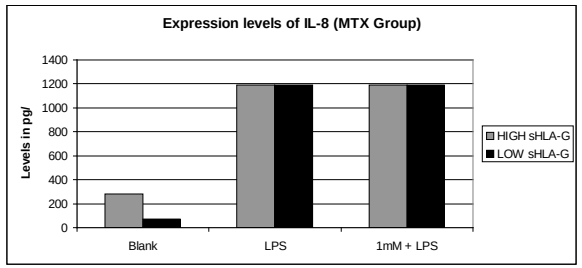
IL-18 Expression differences between the high sHLA-G and low sHLA-G group



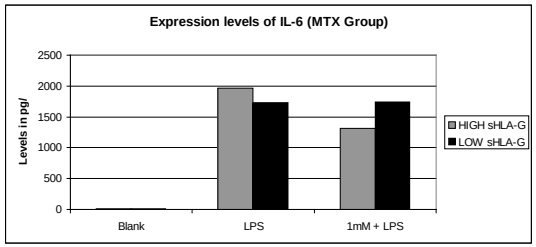
IL-1β Expression differences between the high sHLA-G and low sHLA-G group



TNF-α Expression differences between the high sHLA-G and low sHLA-G group



IL-8 Expression differences between the high sHLA-G and low sHLA-G group



IL-6 Expression differences between the high sHLA-G and low sHLA-G group

Figure 6-10 Differences in the expression levels of the examined cytokines IL-23, IL-18, IL-1β, TNF-α, IL-6 and IL-8 after co incubation 1 μM MTX.

The low sHLA-G and high sHLA-G groups are being compared. MTX (methotrexate), TNF-alpha (Tumour necrosis factor-alpha), Interleukin 23 (IL-23), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interleukin 18 (IL-18), Interleukin 1 -beta (IL-1B)

Table 6-1IL-10 expression in PBMC cultures incubated with 6-MP and the relationship with recognised high producer and low producer haplotypes of the IL-10 promoter.

The IL-10 concentrations are reported in pg/ml. The values reported are all the mean of the duplicate values. Sixteen patients are included in this analysis. The low producer haplotypes are ACC/ACC, ATA/ACC and ATA/ATA. The high and intermediate producers which are grouped together are GCC/ACC, GCC/ATA and GCC/GCC.

IL-10 Haplotype	BLANK	LPS		0.25mM 6-MP		0.5 mM 6-MP		1mM 6 MP		p (0.25 - 1 mM expression levels)
		24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	
All patients	4	518	221	495	155	489	152	522	169	
Low producer IL-10 promoter Haplotypes	4	521	198	521	153	521	132	521	132	p= 0.2
High producer IL-10 promoter haplotypes	4	516	239	475	157	464	167	523	198	

Table 6-2 IL-10 expression in PBMC cultures incubated with methotrexate and the relationship with recognised high producer and low producer haplotypes of the IL-10 promoter.

The IL-10 concentrations are reported in pg/ml. The values reported are all the mean of the duplicate values. Sixteen patients are included in this analysis. The low producer haplotypes are ACC/ACC, ATA/ACC and ATA/ATA. The high and intermediate producers which are grouped together are GCC/ACC, GCC/ATA and GCC/GCC.

IL-10 Haplotype	BLANK	LPS		0.25mM MTX		0.5 mM MTX		1mM MTX		p (0.25 - 1 mM expression levels)
		24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	
All patients	4	518	218	528	179	522	182	517	157	
Low Producer	4	521	243	521	143	521	140	521	96	p= 0.4
High Producer	4	516	230	534	207	523	215	513	204	

Table 6-3 IL-10 expression in PBMC cultures incubated with 6-MP/MTX and the relationship with recognised HLA-G 14 bp ins/del genotypes.

6-MP Group	BLANK	LPS		0.25mM 6-MP		0.5 mM 6-MP		1mM 6 MP		p (0.25 - 1 mM expression levels)
		24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	
All patients	4	518	221	495	155	489	152	522	169	p=0.7
HLA-G 14 bp DEL/DEL	4	540	221	586	134	505	113	586	129	
HLA-G 14 bp ins/del and ins/ins	4	543	204	508	148	519	138	548	172	
MTX Group	**BLANK**	**LPS**		**0.25mM MTX**		**0.5 mM MTX**		**1mM MTX**		**p (0.25 - 1 mM expression levels)**
		24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	
All patients	4	518	218	528	179	522	182	517	157	p=0.9
HLA-G 14 bp DEL/DEL	4	540	213	586	176	586	175	586	143	
HLA-G 14 bp ins/del and ins/ins	4	543	292	557	168	549	172	513	149	

The IL-10 concentrations are reported in pg/ml. The values reported are all the means of the values. Sixteen patients are included in this analysis. The low producer genotype is HLAG 14bp ins/ins and ins/del. The high producer genotype is HLA-G 14bp del/del. The p value reported is of the difference between the IL-10 expression levels between the 0.25 μM and the 1 μM concentration of the drug used.

Table 6-4 The changes in sHLA-G levels between LPS stimulated PBMC samples with or without 6-MP or MTX.

All values are expressed in ng/ml. The drug concentrations are in μM. The HL-G 14bp ins/del genotypes are being compared.

		Un- stimulated	LPS Stimulated						
14bp Genotype		HLA G BLANK	HLA G	1 6MP	0.5 MTX	1 MTX	Change with 1mM 6MP	Change with 0.5mM MTX	Change with 1mM MTX
del/del	**Mean**	0.20	2.49	4.12	4.50	5.02	1.63	2.05	2.53
	Standard Deviation	0.07	0.99	1.58	2.83	1.36	0.80	3.40	1.16
	Number of samples	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
ins/del	**Mean**	0.18	3.37	3.44	4.68	2.83	0.06	1.30	-0.54
	Standard Deviation	0.03	1.62	2.22	3.29	2.11	1.79	3.10	2.96
	Number of samples	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
ins/ins	**Mean**	0.18	3.76	3.29	1.70	1.80	-0.40	-2.00	-1.90
	Standard Deviation	0.02	2.02	1.09	1.10	0.78	1.29	2.20	2.30
	Number of samples	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00

Table 6-5 : The expression levels of the studied cytokines divided into Low and High s HLA-G expressing individuals- 6-MP group.

The drug used is 6-mercaptopurine. All values are expressed in ng/ml

	sHLA-G	SAMPLE	IL-23	IL-8	IL-6	IL-18	IL-1B	TNF-A
6-MP	HIGH	BLANK	77	280	10	46	30	27
		LPS	98	1192	1971	54	80	34
		0.5 μM 6MP	85	415	11	46	30	28
		LPS + 0.5 μM 6MP	100	1089	1994	45	74	30
		1 μM 6MP	76	239	10	45	30	27
		LPS + 1 μM 6MP	81	1191	2021	62	74	46
	LOW	BLANK	78	69	10	44	30	27
		LPS	98	1192	1732	52	52	36
		0.5 μM 6MP	73	161	13	57	31	32
		LPS + 0.5 μM 6MP	79	795	907	33	31	23
		1 μM 6MP	75	127	10	44	30	27
		LPS + 1 μM 6MP	101	1192	1608	44	55	31

All data displayed is for 48 hour incubation. 6-MP (6-mercaptopurine), TNF-alpha (Tumour necrosis factor-alpha), Interleukin 23 (IL-23), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interleukin 18 (IL-18), Interleukin 1 -beta (IL-1B)

Table 6-6 The expression levels of the studied cytokines divided between the High and Low sHLA-G producers- MTX Group.

The drug used is MTX and all values are expressed in ng/ml

	sHLA-G	SAMPLE	IL-23	IL-8	IL-6	IL-18	IL-1B	TNF-A
MTX	HIGH	BLANK	77	280	10	46	30	27
		LPS	98	1192	1971	54	80	34
		0.5 μM MTX	78	295	10	44	30	27
		LPS + 0.5 μM MTX	111	1166	1715	46	60	31
		1 μM MTX	79	263	10	45	30	27
		LPS + 1 μM MTX	91	1192	1314	45	45	29
	LOW	BLANK	78	69	10	44	30	27
		LPS	98	1192	1732	52	52	36
		0.5 μM MTX	70	151	13	54	31	32
		LPS + 0.5 μM MTX	95	1192	1262	45	53	29
		1 μM MTX	81	142	11	47	30	27
		LPS + 1 μM MTX	88	1192	1737	54	59	37

All data displayed is for 48 hour incubation. MTX (methotrexate), TNF-alpha (Tumour necrosis factor-alpha), Interleukin 23 (IL-23), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interleukin 18 (IL-18), Interleukin 1 -beta (IL-1B).

6.6 Discussion

PBMCs were selected as the preferred cell type for study. It would have been ideal to study lamina propria mononuclear cells, but access to the cell population in normal individuals would be difficult. These two cellular populations are not identical and important differences between them can influence the outcomes of certain experiments.

The role of the promoter region (5'UTR) of a gene in mediating transcription of functional mRNA is important and in conjunction with degradation and variations in stability of the translated protein influenced by 3'UTR region, they influence mRNA levels [455]. The HLA-G promoter region is variable and 29 SNPs have been described, some of which appear to correlate with asthma and adverse foetal outcomes. However, sHLA-G levels have not been found to be related to these outcomes and they have been inconsistent with contradicting results being found in different conditions such as eclampsia and multiple sclerosis [165]. The effects of genetic variation in this region are uncertain. A number of polymorphisms have been reported in this area some of which are associated with variant HLA-G expression. Many of these are in LD with the 14bp ins/del polymorphism and include +3142G, +3187A and +3003C [456]. Quite at odds with a variety of functional correlations of variant genotypes, the HLA-G 14bp ins/del and it's associations with foetal outcomes and immunosuppressive medication outcomes are very consistent. [457]

A number of effects of HLA-G are seen on regulatory cells. Dendritic cells (DC) can be induced by the effect of HLA-G1 or HLA-G 5 through the inhibitory ILT receptors. The subsequent down regulation of co stimulatory CD80 and CD86 renders the DCs suppressive or tolerogenic [458]. Furthermore, suppressive CD4+ CD25- CTLA4+ T cells and CD 8+ CD 28- T_{regs} that produce IL-10 are generated. HLA-G1+ APC stimulate and cause the differentiation of T cells into suppressor cells [162]. They cannot be defined phenotypically, and do not secrete HLA-G but instead mediate suppressive effects by the secretion of IL-10 [161]. In contrast, naturally occurring HLA-G+ T_{regs} do not produce IL-10 or TGF- β and are very distinct from Tr 1 / Th 3 cells or induced HLA-G+ T cells[459].

IL-10 production in PBMC cultures has previously been noted to be highest in individuals with the HLA-G 14bp ins/ins genotype [180] and serum IL-10 levels have been implicated in

modifying response to immunomodulators in RA [452]. HLA-G and IL-10 appear to be closely interrelated in their immunological influence [180]. Hence, it is possible that the HLA-G 14bp insertion destabilises mRNA and impairs sHLA-G production. When this is associated with an impaired IL-10 secretion, the response to immunomodulatory drug therapy may be diminished. Although limited by small numbers in sub group analysis, the genotyping data presented here appears to support this hypothesis. The results show that the combination of the favourable HLA-G 14 bp deletion and the IL-10 GCC promoter haplotype favours a clinical response to AZA therapy in IBD patients.

This offers an intriguing explanation for the considerable variability in studies on the association of polymorphisms in the IL-10 promoter and the susceptibility to IBD. The low producer -1082 A/A genotype has been associated with steroid resistance in CD [460] and these experiments have shown that IL-10 expression is influenced by exposure to 6-MP and MTX. It is conceivable that the conflicting results may be due to the pharmacogenetic influence of IL-10 (steroids and 6-MP or MTX) on the disease phenotype.

The functional data in this study demonstrate high levels of sHLA-G generation in mixed lymphocytic cell cultures incubated with 6-MP in individuals with the high sHLA-G producing genotype. This is associated with relatively increased levels of IL-10. However, there are apparent differences between cytokine levels after exposure to 6-MP and those after exposure to MTX *in vitro*. In the MTX group, there are lower levels of some pro inflammatory cytokines. In the 6-MP group, no such differences are seen. Overall, the differences do not amount to significance but are certainly suggestive. They would be consistent with increases in induced T_{regs} in the 6-MP group. T_{regs} are thought to express IL-10 and suppress colitis in mice but the role is less clear in humans. Tr 1 cells are present in higher levels in inflamed colonic mucosa in patients with UC and the administration of IL-10 did not suppress colitis in humans. It is possible that the levels of Tr 1 expression seen are not adequate for the induction of mucosal tolerance. [461]

Hence, it may be that 6-MP, through the peripheral generation of T_{reg} cells, can control and inhibit ongoing inflammation. It may induce IL-10 generating dendritic cells (DC-10) and up-regulate inhibitory ILT receptors on DC. These specifically induce Tr-1 cells that exert an anti inflammatory, suppressive or tolerogenic effect. There is some more support for this hypothesis from recent work in lamina propria T_{regs} and peripheral blood T_{regs} in IBD. Both are low in active disease with heightened levels of apoptosis. Anti TNF-α medication reverse these changes and are

most prominent in individuals with IBD who respond clinically to this medication [462]. T_{reg} activity is clearly involved in the response to anti TNF-α and may well be relevant in other drugs such as AZA/6-MP. In this vein , a recent letter suggests that sHLA-G levels are higher in LPS stimulated PBMC cultures from blood in individuals with UC who are receiving treatment with 6-MP when compared with those who are receiving mesalazine or steroids [463]. As mentioned earlier, the active inflammatory state may well be a confounding factor but taken with the report that MTX responsive RA patients have an induction of IL-10 producing T_{regs} from plasmacytoid DCs [464], the hypothesis certainly appears plausible. This work has also found that IL-10 levels are affected by MTX *in vitro* and are influenced by the IL-10 promoter haplotype.

This work presented here has not evaluated lamina propria cells, but PBMC T_{reg} populations appear to be good surrogates for examining these effects. Potential influences are not easy to study as there are likely to be a number of redundant regulatory mechanisms in intestinal mucosa and as mentioned before, access to both inflammed and non inflammed intestinal tissue is limited [465]. The approach adopted in this work of identifying a genetic marker followed by an attempt at functional correlation *in vitro* sets the stage for a study in LPMCs. The lack of a definitive marker of T_{regs} cells is a limitation, but nevertheless , it would be illuminating to examine the effects of 6-MP, MTX and INFX *in vitro* on both lamina propria and peripheral blood T_{reg} cell populations and investigate an association with sHLAG levels.

7 Conclusions

The primary aim of the study was the detailed evaluation of the pharmacogenetics of MTX therapy in IBD. This thesis has examined clinically relevant polymorphisms in a number of folate pathway enzymes. An important aspect was the study of the pharmacogenetic effects of folate supplementation as well as of haplotypes and compound heterozygosity on clinical response rates and drug intolerance. Such information has been lacking in IBD studies.

In this work, there are some small effects such as *MTHFR 677 TT* genotype which was found more often in clinical responders receiving oral folate and the MTHFR 677 T allele seen more frequently in the same group. The individuals affected by this polymorphism are small (n=19). The expected influence of compound heterozygosity was not seen and instead, the MTHFR 677CT/1298AA haplotype was overrepresented in individuals who experienced all cause side effects. The number of affected individuals is once again small and an assessment of the influence of folate supplementation is difficult to make. The problems with assessing all cause side effects have been explained in a previous chapter. The small number of individuals with the polymorphism and the pooling of all side effects make it difficult to consider a mechanism for this effect. The effect of the 677TT/1298AA haplotype on response rates is completely influenced by the previously described 677 TT polymorphism and has no additional clinical significance.

The TSER tandem repeats and the TS6bp ins/del polymorphisms did not influence clinical response rates or side effects. Folate supplementation had no influence either. The close relationship between MTHFR and TS was examined thoroughly. The interesting hypothesis that the *MTHFR677TT* mutation would impart reduced clinical efficacy on MTX therapy in individuals who possess the TS 2*R with the TS 6bp deletion was not proven and folate supplementation had no influence.

The expectation of an increase in clinical response rates among those with the common *ATIC 347 GG* genotype was fulfilled but once again, the influence was small. It was encouraging that the effect was consistent with recent GWAS results that have provided some clarity to yet another controversial area in pharmacogenetics [481]. Individuals with the wild type ATIC 347 G allele were more likely to be tolerant of MTX therapy. The putative influence of the ATIC enzyme on adenosine levels and subsequent anti inflammatory effects of MTX is not helpful in explaining

this observation. Folate supplementation did not have an effect and although this observation has been reported before, a hypothesis is lacking.

No effects of the polymorphism in the drug transporter Reduced folate carrier and the extracellular oxidative enzyme Aldehyde oxidase were seen. Hence, at the end of the initial experiments, a marker for clinical response or drug intolerance similar to TPMT had not been found. This experience is not unique and TPMT remains the most successful clinical application of pharmacogenetics despite considerable effort. The relative weakness and the limitations in applying successful pharmacogenetic models from cancer chemotherapy to inflammatory diseases are easily evident.

There are a number of reports of varying cytokines profiles and hence, an implied variability in adaptative immunity and the effects these have on response to MTX and 6-MP therapy. There have also been many attempts to find identify polymorphisms in the relevant genes that could predict such effects. However they have been less encouraging than the classical pharmacogenetic approach examining drug metabolism. In this setting, the reports on the role of the HLA-G complex in tolerance are very impressive. It has been extensively evaluated in foetal medicine, cancer cell biology and post organ-transplant immunosuppression. It builds well towards an application of a 14bp insertion/deletion polymorphism in predicting clinical response to immunonodulation. This polymorphism could be a marker of T cell regulatory activity and a pharmacogenetic marker of the influence of T regulatory activity in IBD is attractive. Consistent with the results from Rheumatoid arthritis, we found a powerful influence of the 14 bp insertion polymorphism on impaired clinical response to methotrexate in a large group of IBD patients. As expected, the influence was not affected by folate supplementation. The number of individuals affected by UC was not large but there were no discernible difference in response rates between individuals with UC or CD.

This brought up a number of important issues for consideration. The proposed mechanism of the effect of the polymorphism on immunomodulatory therapy was likely to be true for a number of drugs and this has been reported in renal and cardiac transplantation experience. However, our experiment showed that the 14 bp insertion was not over represented in individuals who arrived at MTX therapy after AZA/6-MP failure. It was possible that the influence would be greater in individuals with UC as intestinal epithelial cell HLA-G expression was higher in UC than in CD. The HLA region has a high level of LD and this region has known associations with CD

susceptibility and the 14bp polymorphism was associated with ileal disease in individuals with CD. The precise mechanism was unclear but there were reports of high IL-10 expression levels in those with the 14bp ins/ins genotype and it was likely that common IL-10 promoter polymorphisms had a secondary influence on the observed effects.

Each of these was examined in turn. First, it is demonstrated that the 14 bp polymorphism had no influence on susceptibility to IBD (CD or UC) in a large cohort of patients when compared with normal controls. The DNA was obtained from a previously reported cohort. The results from that cohort have been validated in a number of publications. There was no association with disease location in CD or UC and it is likely that the polymorphism does not affect disease severity in CD.

Next, the role of the HLA-G 14 bp ins/del polymorphism in clinical response to AZA in patients from a previously reported large multicenter trial [263] of the use of this drug in IBD was examined. The effect was very prominent on response rates and consistent with that seen in the MTX group. It was also demonstrable that there was a clear difference between the UC group and CD groups. The genotype was distributed equally between the UC and CD group, but when the groups were stratified for clinical response, the effect was more prominent in the CD group. This appears at odds with reports that HLA-G expression is greater in intestinal epithelium from individuals with UC and hints at a mechanistic influence that is currently unclear. The influence of the TGN levels, TPMT genotype, concomitant medication, age and sex were examined. The TPMT genotype correlated with clinical response in the study group, but the effect was less prominent than that of the 14bp genotype.

The effect of three common and well described polymorphisms of the IL-10 promoter gene on clinical response rates to AZA and on an additive influence with the HLA-G 14 bp polymorphism was investigated. The favourable and anti inflammatory *IL-10* promoter GCC haplotype was not associated with clinical response rates with either AZA or MTX therapy. However, possession of both the favourable 14bp del/del genotype and the *IL-10 GCC* haplotype conferred a significant likelihood of clinical response compared to those who lacked this combination on AZA therapy. The numbers are small and a clear conclusion is difficult to make. In the larger MTX group , a conditional analysis on the influence these two polymorphisms may have on each other and on clinical response show that the haplotype of HLA-G14bp del and *IL10* -1082G is strongly associated with clinical response but this is primarily from the HLA-G14bp del contribution.

However, an examination of the OR suggests that the favorable IL10-1082 G allele appears to reduce the risk of therapeutic failure on MTX therapy in those with the HLAG 14bp ins allele.

The HLA-G 14bp ins/del polymorphism influences soluble HLA-G levels and higher sHLA-G levels correlate with greater tolerance. The effect of pharmacologically relevant concentrations of MTX and 6-MP on the levels of sHLA-G in monocyte cultures was examined. These changes were correlated with genotype and a clear influence was seen in both the 6-MP and the MTX cultures. The favorable HLA-G 14bp del/del genotype produced the highest levels of sHLA-G and the unfavorable HLA-G 14bp ins/ins genotype produced the lowest levels after 48 hours of incubation. A dose effect was seen and the changes were not evident at the lower doses studied. This appears to support the findings from the genetic experiments. An important observation is that normal volunteers were recruited for these experiments and PBMCs rather than LPMCs were examined. This was because of the large number of confounding variables that active systemic inflammation in individuals such as those with IBD would have introduced to these experiments. It would have been more difficult to procure LPMCs form normal individuals. A study of the effects in LPMCs from IBD patients would be a logical extension of these experiments and could be the focus of future work.

As there appears to be an influence of IL-10 promoter haplotype on the effect of the HLA-G 14bp genotype on clinical response to MTX and perhaps to 6-MP, the interaction between IL-10 levels, HLA-G genotype and IL-10 promoter haplotypes were examined. None of the examined hypotheses reached significance, however interesting trends were seen. There was a dose effect of 6-MP on IL-10 levels with an increase in IL-10 levels with increasing 6-MP concentrations in the culture medium. The high producer haplotypes produce more IL-10 on co incubation with both 6-MP and MTX. The HLA-G 14bp ins/del genotypes did not influence IL-10 levels at baseline or on co incubation with varying concentrations of 6-MP or MTX. Hence although there appeared to be some moderating influence of IL-10 promoter haplotypes on the pharmacogenetic effects of the 14bp genotype in MTX therapy, there were no such effects seen on sHLA-G levels. The study population may have been too small to reveal this effect. Alternatively, it may be that there was an influence, but that was modified by other cytokine level changes.

Although significance is not achieved, there are suggestive differences in the levels of inflammatory cytokines between the high sHLA-G and low sHLA-G groups when the LPS stimulated PBMCs are exposed to MTX. The pro-inflammatory cytokines appear to be

suppressed. Similar effects are not seen with 6-MP suggesting an alternative mechanism perhaps more predisposed towards expression of the anti inflammatory IL-10.

There is scope for further work in this field. The functional studies could be expanded and Lamina propria monocytes could be used. This would be a closer approximation of the mechanisms at work *in vivo*. The cytokine profiles could be refined further and may identify those individuals who are more likely to respond to other medication namely Infliximab. Once such influences are clearly defined, the next step would be the application of the pharmacogenetic marker to a prospectively recruited group of individuals with IBD. They would then be exposed to defined therapeutic algorithms and their clinical response would be correlated both to genotype and serum sHLA-G levels. The levels could be examined sequentially over time and correlations made with drug response as well as the subsequent loss of response to therapy. Attempts would be made to identify those predisposed to therapeutic failure and suited to early anti TNF therapy or even surgery to minimise drug induced complications.

The work presented in this thesis makes an interesting case for the application of a pharmacogenetic marker of response to immunomodulatory therapy in patients with IBD that departs from the traditional approach. This marker for tolerance of an inflammatory state appears to identify those predisposed to respond to immunosuppressive medication. It is recognized that the response to medication is not usually predicted by single genetic loci. Instead, a polygenic approach that includes multiple loci and the interaction between these is proposed as a viable alternative. Nevertheless, the clinical application of such pharmacogenetic panels or indices has been disappointingly unimpressive. Clearly there are other influences at work. There are many possibilities and they include variations in metabolizing enzymes, drug transporters, receptors, drug targets, absorption mechanisms, excretory mechanisms, confounding interactions between drugs etc. Even the impressive advances in high throughput genotyping with micro array based and sequencing technology is not adequate to cover all of the mechanisms that are of interest. The inclusion of markers of hitherto unexplored influences on drug response may help achieve the ideals of pharmacogenetics.

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Appendices

Appendix 1 : Consent Forms

Consultant Gastroenterologist: Dr Jeremy Sanderson
Investigator: Dr Melissa Smith/Dr Bijay Baburajan
Contact No. 02071882497
LREC Study No. 06/Q0707/84
Patient ID No.

Consent Form for: Pharmacogenetics of immunomodulatory treatment in inflammatory bowel disease

1. I confirm that I have read and understood the information sheet (version dated May.2008) about this project and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my medical notes may be looked at by medically qualified staff from Guy's and St Thomas' Hospitals.
I give permission for these individuals to have access to my records. ☐

4. I understand that my GP will be contacted to inform them that I am participating in this study. ☐

5. I agree to take part in the above study ☐

_____	_____	_____
Name of patient	Signature	Date
_____	_____	_____
Name of researcher	Signature	Date

1 copy for patient, 1 copy for researcher, 1 copy for notes.
Version 1, May. 2008

Appendix 2: GP Information Sheet

GP information Sheet
Pharmacogenetics of immunomodulatory treatment in IBD

Dear Doctor,

We are writing to let you know that your patient:_____
has agreed to take part in our research study as a subject who does not have inflammatory bowel disease and is not any immunomodulatory treatment.

The aim of this study is to find out why it is that some people respond so well to immunomodulatory treatment, whilst others have no response or experience adverse events.

Our study simply involves taking a blood sample from your patient for an in-vitro experiment. The relevant WBCs will be extracted and incubated with methotrexate and 6-mercaptopurine. The samples will then be studied for the cytokine expression profiles and correlation will be made with genotypes of certain genes influencing cytokine function.

As well as testing patients currently taking these medications we are also interested in those patients who have had them in the past, but had to stop them due to adverse events or because they were ineffective.

Your patient's co-operation with our trial will not affect their ongoing treatment at GSTT in any way and should not have any impact on their care in General Practice.

If you have any questions about our study then please feel free to contact us on 02071882497.

Yours faithfully,

Dr Jeremy Sanderson

Version 1 May 2008

Appendix 3 :Patient Information Sheet

Patient information Sheet
Pharmacogenetics of immunomodulatory treatment in Inflammatory Bowel Disease

You are being invited to take part in a research study. Before you decide whether or not you wish to be involved it is important for you to understand why the research is being done and what it will involve for you. If you have any questions please ask. You can take your time to decide if you want to take part and talk to others about it if you wish.

What is the purpose of this study?

Inflammatory bowel disease is a condition that affects many people, young and old. It contributes to considerable ill health to those affected and can sometimes be fatal. Many people require operations to get better. Whilst we know that immunomodulatory medications (such as azathioprine (AZA), 6-mercaptopurine (6MP), methotrexate, cyclosporin and infliximab) are effective treatments for IBD but they don't work for everyone and can cause side effects. This may be due to differences in the way that people's bodies break down these drugs which is controlled genetically. By analysing blood samples from people who do not have these diseases and who are not on these medicines , we can tell how the normal human body is metabolising them and look directly at differences in the genes responsible for this process.

Why have I been chosen?

You are being chosen for this study because you do not suffer from these diseases and you are not on the medicines whose effects we are trying to study. After the sample has been taken, your blood cells can be analysed for their response to these medicines and that response can be correlated to your genetic makeup

Do I have to take part?

No. It is up to you to decide whether or not to take part and whatever you decide your treatment will be unaffected. You are free to withdraw from the study at any time.

What will happen to me if I take part?

You will need to sign a consent form and then be asked to provide approximately 18mls of blood (4 teaspoons) in addition to that taken for your normal clinic tests. In order for us to relate the blood results to how you are responding to your treatment you will also be asked to answer a few quick questions about your treatment and how active your IBD is at present. No change will be made to your treatment and you will not need to make any extra trips to the clinic.

What are the risks of taking part?

There is no risk to your health or safety from taking part in this study. The type of genetic information that we will get from testing your blood would not affect your insurance status or have implications for your future health or that of your family. The results of any genetic tests will also be anonymous in order to further protect you.

What are the benefits of taking part?

We cannot promise that the study will help you directly but the information we get might help improve the treatment of people with Crohn's and UC in the future.

What happens when the study stops?

All remaining blood samples will be destroyed. We will let all participants know the results of our study.

Will my taking part in the study be kept confidential?

Yes, except that it is considered good practice for us to alert your GP that you have volunteered to help us and to send them a similar sheet to this, explaining what we are doing. Authorised researchers will need to look at your medical records to collect information about treatments that you have taken and how you have responded to them. Nothing that could reveal your identity will ever be published or released from the study. Our procedures for handling, processing, storage and destruction of patient information are compliant with the Data Protection Act 1998.

What will happen to the results of the research study?

We hope that the study will provide interesting results that improve treatment for people with Crohn's and UC. We plan to publish the study's results in medical journals. In any publication of the study's results you will not be identifiable unless your consent has been specifically sought for this.

Who is organising and funding the research?

This research is being organised and funded by Guys and St Thomas' Hospital Trust. We have also applied to the National Association for Crohn's and Colitis (NACC) for financial support. There is no other outside sponsorship or incentive payment to researchers for including you in the study. This study has been approved by the Bexley and Greenwich Local Research Ethics Committee

What if there is a problem?

Any complaint about the way in which you have been treated during the study will be addressed. Researchers are all members of Trust Staff and are covered for negligent harm via the usual arrangements.

You will be given a copy of this sheet and your consent form. Thank you for taking the time to read this information sheet and for considering taking part in our study.

Contact Details:
If you have any additional questions about our study please feel free to contact us on this number: 02071882497

Version Two May 2008

Appendix 3 :Patient Information Sheet To be posted to the patient before attending at the Hospital

Dear ……

You have been offered an appointment to attend at Guy's and St Thomas' Hospital for an appointment. We are a teaching hospital and are involved in the research of a number of disease conditions. We would like to invite you take part in one of these studies.

Before you decide whether or not you wish to be involved it is important for you to understand why the research is being done and what it will involve for you. If you have any questions please ask. You can take your time to decide if you want to take part and talk to others about it if you wish.

What is the purpose of this study?

Inflammatory bowel disease is a condition that affects many people, young and old. It contributes to considerable ill health to those affected and can sometimes be fatal. Many people require operations to get better. Whilst we know that some medicines are effective treatments for IBD , they don't work for everyone and can cause side effects. This may be due to differences in the way that people's bodies break down these drugs which is controlled genetically. By analysing blood samples from people who do not have these diseases and who are not on these medicines, we can tell how the normal human body is metabolising them and look directly at differences in the genes responsible for this process.

Why have I been chosen?

You are being chosen for this study because you do not suffer from these diseases and you are not on the medicines whose effects we are trying to study. After the sample has been taken, your blood cells can be analysed for their response to these medicines and that response can be correlated to your genetic makeup

Do I have to take part?

No. It is up to you to decide whether or not to take part and whatever you decide your treatment will be unaffected. You are free to withdraw from the study at any time.

What will happen to me if I take part?

You will need to sign a consent form and then be asked to provide approximately 18mls of blood (4 teaspoons) in addition to that taken for your normal clinic tests.

No change will be made to your treatment and you will not need to make any extra trips to the clinic.

What are the risks of taking part?

There is no risk to your health or safety from taking part in this study. The type of genetic information that we will get from testing your blood would not affect your insurance status or

have implications for your future health or that of your family. The results of any genetic tests will also be anonymous in order to further protect you.

What are the benefits of taking part?

We cannot promise that the study will help you directly but the information we get might help improve the treatment of people with Inflammatory Bowel Disease in the future.

Will my taking part in the study be kept confidential?

Yes, except that it is considered good practice for us to alert your GP that you have volunteered to help us and to send them a similar sheet to this, explaining what we are doing. Authorised researchers will need to look at your medical records to collect information about treatments that you have taken and how you have responded to them. Nothing that could reveal your identity will ever be published or released from the study. Our procedures for handling, processing, storage and destruction of patient information are compliant with the Data Protection Act 1998.

What will happen to the results of the research study?

We hope that the study will provide interesting results that improve treatment for people with Inflammatory Bowel Disase. We plan to publish the study's results in medical journals. In any publication of the study's results you will not be identifiable unless your consent has been specifically sought for this.

Who is organising and funding the research?

This research is being organised and funded by Guys and St Thomas' Hospital Trust. There is no other outside sponsorship or incentive payment to researchers for including you in the study. This study has been approved by the Bexley and Greenwich Local Research Ethics Committee

What if there is a problem?

Any complaint about the way in which you have been treated during the study will be addressed. Researchers are all members of Trust Staff and are covered for negligent harm via the usual arrangements.

You will be given a copy of this sheet and your consent form. Thank you for taking the time to read this information sheet and for considering taking part in our study.

Contact Details:

If you have any additional questions about our study please feel free to contact us on this number: 02071882497

Dr Jeremy Sanderson
Consultant Gastroenterologist and Chief Investigator

NOTICE OF SUBSTANTIAL AMENDMENT

For use in the case of all research other than clinical trials of investigational medicinal products (CTIMPs). For substantial amendments to CTIMPs, please use the EU-approved notice of amendment form (Annex 2 to ENTR/CT1) at http://eudract.emea.eu.int/document.html#guidance.

To be completed in typescript by the Chief Investigator in language comprehensible to a lay person and submitted to the Research Ethics Committee that gave a favourable opinion of the research ("the main REC"). In the case of multi-site studies, there is no need to send copies to other RECs unless specifically required by the main REC.

Details of Chief Investigator:

Name:	Dr Jeremy Sanderson
Address:	Dept of Gastroenterology, First floor, College House, South Wing, St Thomas' Hospital, Lambeth Palace Road, LONDON, SE1 7EH
Telephone:	0207188 2498
Email:	jeremy.sanderson@kcl.ac.uk
Fax:	0207188 2484

Full title of study:	Pharmacogenetics of immunomodulatory treatment in Inflammatory Bowel Disease.
Name of main REC:	Bexley & Greenwich Local Research Ethics Committee
REC reference number:	06/Q0707/84
Date study commenced:	Feb 2007
Protocol reference *(if applicable)*, current version and date:	Version 3, May 2008

Type of amendment (indicate all that apply in bold)

(a) Amendment to information previously given on the NRES Application Form

No

If yes, please refer to relevant sections of the REC application in the "summary of changes" below.

(b) Amendment to the protocol

Yes

If yes, please submit <u>either</u> the revised protocol with a new version number and date, highlighting changes in bold, <u>or</u> a document listing the changes and giving both the previous and revised text.

(c) Amendment to the information sheet(s) and consent form(s) for participants, or to any other supporting documentation for the study

YES

If yes, please submit all revised documents with new version numbers and dates, highlighting new text in bold.

Is this a modified version of an amendment previously notified to the REC and given an unfavourable opinion?

No

Summary of changes

We will recruit patients with inflammatory bowel disease and controls (patients without IBD, cancer, Inflammatory joint diseases, skin diseases, SLE, who are not pregnant or have not had a transplanted organ. They will not be on any immuno modulatory medication). In addition to the blood sample for genotyping(which we already have permission to collect), we would also like to collect blood samples to study the effects of azathioprine , 6-mercaptopurine, methotrexate, Infliximab, 6 thioguanine and ciclosporin on cytokine expression by white blood cells *in vitro.* This will allow us to look in more detail at the effects of the immunomodulatory drugs on the immune system in health and contrast these with patients with IBD. It is important to study normal patients as many of the diseases and the medications themselves influence cytokine expression and can make results impossible to interpret. We will also study the same samples for the levels of by products of drug

metabolism, giving functional information (to complement the genetic data we are already collecting). This will strengthen the conclusions that we are able to draw from our results.
As anticipated when we submitted our original application, additional target genetic loci have become relevant since our initial protocol was drawn up and we would also like to test our blood samples for genetic variation in HLA-G, IL-10, IL23R and other cytokine genes.

Any other relevant information

We do not anticipate any additional ethical issues to arise. As with our original protocol, we are not altering the patient's care or medication in any way, simply collecting data and samples during a routine outpatient appointment.

Declaration

- I confirm that the information in this form is accurate to the best of my knowledge and I take full responsibility for it.
- I consider that it would be reasonable for the proposed amendment to be implemented.

Signature of Chief Investigator: ……

Print name: Dr Jeremy Sanderson

Date of submission: 23.05.08

Differences in Cytokine levels between low producer and high sHLA-G producer individuals after exposure *in vitro* **to 6-mercaptopurine (data used for calculation of significance).**

All cytokine levels displayed are the difference between the LPS stimulated sample and the named study sample. All data displayed is for 48 hour incubation. 6-MP (6-mercaptopurine), TNF-alpha (Tumour necrosis factor-alpha), Interleukin 23 (IL-23), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interleukin 18 (IL-18), Interleukin 1 -beta (IL-1B).

	Samples	TNF-α		IL-23		IL-8		IL-6		IL-18		IL-1β	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
High Producer s-HLAG	No Drug (LPS - no LPS)	6.6	9.07	21.3	23	911	450	1960	428	8	19	50	46
	Blank - 0.5 μM 6-MP	-0.6	0.5	-8	3.6	-135	222	0	0	0	2	0	0
	LPS- 0.5 μM 6-MP + LPS	4	7.8	-2	18.7	102	109	-23	39	9	17	6	6
	Change after 6-MP + LPS	4	8	6	17	238	239	-23	40	9	20	6	6
	Blank - 1 μM 6-MP	0.3	0.5	0.6	1.15	41	79	0	0	1	2.6	0	0
	LPS - 1 μM 6-MP + LPS	-12	25.5	16.6	18.7	0.3	0.5	-50	87	-8	35	6.6	33.8
	Change after 6-MP + LPS	-13	26	16	18	-41	80	-50	87	-9	37	7	34
	Samples	TNF-α		IL-23		IL-8		IL-6		IL-18		IL-1β	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Low producer s-HLAG	No Drug (LPS - no LPS)	9	6.5	20	27.8	1123	44	1722	841	8	14	22.6	8
	Blank - 0.5 μM 6-MP	-4.6	8.9	4.3	4.5	-92	114	-2.3	4.04	-12.3	20.5	-1	1.7
	LPS- 0.5 μM 6-MP + LPS	13	18.08	18.3	89	397	688	825	1213	19.3	25.9	21.3	33.6
	Change after 6-MP + LPS	18	26	14	90	489	711	828	1217	32	43	22	35
	Blank - 1 μM 6-MP	0.6	0.5	3	12.5	-57	93	-0.3	0.5	-0.3	2.8	0	0
	LPS - 1 μM 6-MP + LPS	5	8.8	-3.6	9.4	0	0	123	214	8.6	15.14	-2.6	9.2
	Change after 6-MP + LPS	4	9	-6	22	58	93	124	214	9	12	-3	9

Cytokine levels between low producer and high sHLA-G producer individuals after exposure *in vitro* **to methotrexate (data used for calculation of significance).**

Cytokine levels are expressed as the difference in either stimulated or un-stimulated samples. Comparison is made between samples with or without study medication. All data displayed is for 48 hour incubation. MTX, methotrexate, TNF-alpha (Tumour necrosis factor-alpha), Interleukin 23 (IL-23), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interleukin 18 (IL-18), Interleukin 1 -beta (IL-1B)

	Samples	TNF-α		IL-23		IL-8		IL-6		IL-18		IL-1β	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
High Producer s-HLAG	No Drug (LPS - no LPS)	6.6	9.07	21.3	23	911	450	1960	428	8	19	50	46
	Blank -0.5 μM MTX	0.5	0.7	-2	2.8	114	173	0	0	2	1.4	0	0
	LPS - 0.5 μM MTX + LPS	3.3	10.6	-13	15.7	26	45	255	442	8.3	16.65	20.6	41.6
	Change after MTX + LPS	3	11	-12	15	-50	95	255	442	7	18	21	42
	Blank- 1 μM MTX	1	0	-2.3	10.2	17	28	0	0	1	1	0	0
	LPS - 1 μM MTX + LPS	4.6	9.8	7.3	13.5	0	0	657	578	9.3	19.7	35	41
	Change after MTX + LPS	4	10	10	15	-17	29	657	578	9	19	35	41
	Samples	TNF-α		IL-23		IL-8		IL-6		IL-18		IL-1β	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Low producer s-HLAG	No Drug (LPS - no LPS)	9	6.5	20	27.8	1123	44	1722	841	8	14	22.6	8
	Blank -0.5 μM MTX	-4.3	9.2	7.3	20.5	-82	164	-2	4.3	9.3	18.7	-0.6	1.1
	LPS - 0.5 μM MTX + LPS	7.3	8.5	2	16	0	0	227	227	10	13	-0.6	9.5
	Change after MTX + LPS	12	12	-5	31	82	164	229	227	19	23	0	9
	Blank- 1 μM MTX	-0.3	0.5	-3	15	-73	144	-0.6	1.15	-2.3	2.08	0	0
	LPS - 1 μM MTX + LPS	-0.6	20	9	33	0	0	-4.3	7.5	-2	27	-7	27
	Change after MTX + LPS	-1	21	12	35	73	145	-4	8	0	29	-7	27